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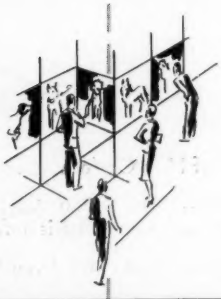

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REFERENCES: 1. Mosier, J. E., and Coles, E. H.: Vet. Med. 53:649 (Dec.) 1958. 2. Belloff, G. B.: Calif. Vet. 9:27 (Sept.-Oct.) 1956. 3. Mosier, J. E.: Vet. Med. 52:445 (Sept.) 1957.

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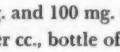

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The Pathogenesis of Hog Cholera
I. Route of Entrance of the Virus into the Animal Body

H. W. DUNNE, D.V.M., Ph.D.; J. F. HOKANSON, D.V.M.; A. J. LUEDKE, D.V.M.

University Park, Pennsylvania

THE INCREASING TREND shown in the past few years toward the eradication of hog cholera has prompted new interest in the methods by which the disease is spread. The discovery, by Shope,[7] of an existing reservoir of hog cholera virus in the egg of the swine lungworm has been an important step in the understanding of the epizootiology of hog cholera and could explain some of the "spontaneous" appearance of the disease in a herd, usually in a single animal.

Once the infection is established in an animal, by direct transmission through contaminated feed, water, or air, there usually occurs a short period between the occurrence of clinical signs of disease in that animal and the subsequent evidence of infection of the herd. Transmission to the rest of the herd in these instances is probably attained by direct contact or indirectly through contaminated feed. The sites of virus invasion in direct transmission, however, have never been established definitely.

It generally has been conceded that, in the direct transmission of hog cholera, the virus usually enters the animal's body during the processes of eating or breathing. Schwarte[5] demonstrated that infection could take place through the respiratory system by passing liquid and powdered aerosols of hog cholera virus through the nasal orifices during the inspiratory phase of respiration or by injecting the powdered virus under pressure through a needle into the trachea. Animals so exposed sickened and died.

Evidence has been presented by Bodian[4] that poliomyelitis virus gains entrance into the human body through the tonsillar area. Since it is generally known that swine can be infected by eating contaminated feed, the question arises as to the importance of the tonsillar area in the process of infection. There are no data available regarding hog cholera virus invasion through the stomach or the tonsillar area. The injection of an aerosol into the trachea does not eliminate the possibility of exhaling the virus past the tonsillar area with subsequent tonsillar infection.

To clarify these points, experiments were designed to determine if hog cholera virus could invade the animal's body through the tonsillar area, the stomach, or the respiratory tract.

MATERIALS AND METHODS

Thirty pigs, weighing 30 to 35 lb., were used. They were hog cholera-susceptible and from nonimmunized sows. The virus used was the "Sprankle" virus, which was isolated from a natural infection of hog cholera in Pennsylvania and which contained 10^6 l.d.$_{50}$ for swine. For the purposes of the gastric exposure, the virus (as undiluted blood) was used in dilutions of 1×10^0 through 1×10^{-6}. A 1×10^{-1} dilution in Tyrode's solution was used in the respiratory and tonsillar exposures.[6]

In the gastric exposures, 19 pigs in four separate tests were given 0.5 ml. of virus in different dilutions placed in a 00 gelatin capsule, which in turn was placed into a 000 gelatin capsule. Care was taken to make sure that no virus contacted the outside of either capsule during the filling process. The capsules were administered with a balling gun, after which the animals were placed in individual

From the Department of Veterinary Science, Pennsylvania State University, University Park, Pa. Authorized for publication on Jan. 2, 1959, as journal series No. 2333 of the Pennsylvania Agricultural Experiment Station, University Park.

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Horsfall-type isolation units for seven days. During this period, strict isolation was maintained. Animals were not handled, and water and feed were introduced through openings which provided a minimum of exposure to possible outside infection.

The preparations of animals for tonsillar and respiratory exposures to the virus were identical. The anesthetic* was injected intravenously at the rate of 1 ml. of a 4 per cent solution per 4 lb. of body weight. The trachea of each animal was exposed by surgery, severed, and the cranial end of the caudal portion was sutured to the skin surface. A tracheal tube was inserted into this portion of the trachea, and the incision was closed tightly to prevent the exposure of the incised edges. The incision and all incised surfaces were sprayed with a sterile liquid surgical dressing (Aeroderm,† co-polymers of hydroxy-vinyl chloride acetate and modified maleic rosin ester) to prevent possible contamination of the incised areas during the period of exposure to the virus. Inhalation of the sprayed surgical dressing was prevented by inserting a cotton pledget into the tracheal tube during the brief periods of application. Infection through areas other than the lungs was avoided by using a sterile sheet over the animal while the virus aerosol was being introduced into the tracheal tube. All animals were exposed under a stainless steel hood with negative pressure for the removal of free aerosol particles, thereby reducing to a minimum the chances of exposure of the animal by other than the intended route. Exposure of the tonsillar area was made by using a 5 by 15 mm. tight sterile cotton swab on a wooden applicator, moistened by diluted infectious blood. Excess blood was squeezed from the swab. With the animal still under anesthesia, the pig's mouth was opened with a speculum, its tongue depressed with a wooden tongue depressor, and the infectious material applied to both tonsillar areas with the swab (fig. 1).

The respiratory exposure was accomplished by introducing a glass tube through a hole in the drape into the tracheal tube. The glass tube was attached by a rubber connection to a DeVilbiss No. 640 atomizer.* Under approximately 3 lb. of pressure, a small amount of the virus aerosol was introduced into the trachea just prior to each inspiration for a total of ten inspirations (fig. 2). The total amount of virus used in the full exposure was less than 1 cc. of a 1×10^{-1} dilution of infected blood. The infective dose was therefore less than 10^{2} m.l.d.

After exposure, the pigs were placed in individual dust-free isolation pens, and provided with water but not feed for the period of survival, which did not exceed 54 hours. Feed was withheld to prevent dust inhalation and to reduce the possibility of irritation of the throat area by contact with trough edges. The animals died from suffocation, as the isolation procedures prevented handling them to clear the air passages in the tracheal tubes. Death occurred in eight to 54 hours, and virus invasion was detected by heart blood samples which were taken at the time of death. Blood specimens were citrated and injected into susceptible pigs which also were maintained in strict isolation. The

TABLE 1—Gastric Exposure of Pigs to Hog Cholera Virus

Pig No.	Source No.*	Virus dilution	Reaction	Challenge (21 days) virus 593			
				Incubation period (days)	Course of illness (days)	Lesions	Termination
669	A593	1×10^{-8}	None	4	50	Severe	Died
670	A593	10^{-7}	None	3	7	Mild	Died
671	A593	10^{-6}	None	2	33	Mild	Died
672	A593	10^{-5}	None	1	53	Moderate	Died
673	A593	10^{-4}	None	1	54	Severe	Died
703	A593	1×10^{-3}	None	3	28	Moderate	Died
704	A593	10^{-5}	None	3	38	Moderate	Died
705	A593	10^{-4}	None	2	45	Severe	Died
706	A593	10^{-3}	None	3	42	Severe	Died
707	A593	10^{-2}	None	2	17	Severe	Died
708	A593	Control	None	3	53	Moderate	Died
722	A591	1×10^{-5}	None	3	13	Severe	Died
723	A591	10^{-4}	None	3	10	Severe	Died
724	A591	10^{-3}	None	4	40	Severe	Died
725	A591	10^{-2}	None	3	32	Severe	Died
726	A591	10^{0}	None	3	52	Severe	Died
748	A593	1×10^{0}	None	2	15	Severe	Died
749	A593	10^{0}	None	2	12	Moderate	Died
750	A593	10^{0}	None	3	11	Severe	Died
751	A593	10^{0}	None	2	14	Moderate	Died

* Identification of pigs which were the source of experimental virus.

* Surital Sodium, produced by Park Davis and Co., Detroit, Mich.

† Aeroderm is produced by Aeroplast Corp., Dayton, Ohio.

* Manufactured by the DeVilbiss Co., Somerset, Pa.

Fig. 1—Tonsillar area exposure to hog cholera virus using a virus-moistened cotton swab.

occurrence of typical hog cholera in these pigs was accepted as positive detection of the virus.

In the absence of clinical signs of hog cholera which could be observed without handling the pigs, all exposed animals were challenged at 21 days with known virulent virus to determine susceptibility.

Lack of immunity to the standard challenge dose of 10^6 l.d.$_{50}$ at 21 days after gastric exposure was accepted as evidence that the virus was not infective by this route.

RESULTS AND DISCUSSION

None of the 19 pigs (table 1) exposed by the gastric route became infected with hog cholera. All proved to be susceptible to hog cholera upon challenge 21 days after the initial (gastric) exposure. It appeared possible that the hydrochloric acid concentration of the stomach was too great to permit survival of the virus. Alteration of the virus presumably was so complete that immunity to the standard challenge dose of 10^6 l.d.$_{50}$ of blood virus was not developed by any of the virus portions which may have been adsorbed into the digestive tract.

Fig. 2—Respiratory exposure with sheet removed for photographic purposes. The aerosol of virus was released momentarily before each inspiration.

It appeared, however, that pigs given infected blood at dilutions of 1×10^{-4} or lower reacted quicker and developed more severe lesions upon subsequent challenge than did those exposed to higher dilutions of the virus. Using the degree of hemorrhage and swelling of the lymph nodes, and hemorrhage of the kidneys and skin as the criteria for severity, 10 of 12 animals given a 1×10^{-4} or lower dilution of virus-infected blood by the gastric route showed severe reaction to subsequent challenge. The other 2 animals showed a moderate reaction. Of those given a 1×10^{-5} or greater dilution of the virus-infected blood by the gastric route, only 2 of 7 showed a severe reaction to subsequent challenge. Of the remainder, 3 pigs showed a moderate reaction and 2 a mild reaction to challenge.

TABLE 2—Respiratory Exposure of Pigs to Hog Cholera Virus

Pig No.	Survival time (hours)	Blood infectivity*
790	30	Positive
840	24	Positive
896	8	Negative
892	21	Negative
969	30	Positive
970	30	Positive

* Infectivity at time of death.

The course of the disease in the challenged pigs ranged from seven to 54 days. The development of increased severity of lesions in the group given the greatest gastric exposure lends emphasis to earlier reports [6] that exposures of pigs to apparently nonimmunizing virus antigens tend to sensitize the animals to later infection with virulent virus. Such animals, upon becoming ill, develop more severe lesions and often more acute courses than animals not previously exposed to nonimmunizing antigen particles.

Somewhat similar but unpublished results with attempts at gastric exposure have been observed by Kernkamp,[7] who used a stomach tube method of exposure but also failed to infect animals by this route. These observations further confirm the findings reported in this paper.

In contrast to the negative results in attempting to infect by the gastric route, exposures of pigs by both the tonsillar route and the respiratory route were successful. Although the pigs with tracheal tubes survived for only a short time, it appeared

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that survival for 24 hours was sufficient to establish viremia. If blood from these pigs produced hog cholera when injected into susceptible pigs, it was considered positive evidence of viral invasion. The complete severing of the trachea and the use of a tracheal tube prevented the possibility of tonsillar infection in intratracheally exposed pigs and prevented respiratory infection in tonsillar-exposed pigs.

Of 6 pigs exposed by the respiratory route, 4 survived for 24 hours or longer (table 2). Blood from the hearts of each of

TABLE 3—Tonsillar Exposure of Pigs to Hog Cholera Virus

Pig No.	Survival time (hours)	Infectivity*
789	24	Positive
792	<22	Negative
977	24	Positive
978	54	Positive

* Infectivity at time of death.

these 4 pigs produced hog cholera when inoculated into susceptible pigs. The other 2 pigs died eight and 21 hours after exposure and their heart blood did not produce hog cholera in susceptible pigs. This indicated that a viremia from respiratory infection sufficient to produce hog cholera in a subinoculate was not established at 21 hours but was established by 24 hours.

Pigs infected by the tonsillar route reacted in a similar manner. Three of 4 pigs infected by this method survived for 24 hours or longer and contained virus in their heart blood at the time of their death (table 3). The other pig died in less than 22 hours after infection. The exact time of death (during the night) was not known. Heart blood from this animal did not produce hog cholera in a susceptible pig.

The short survival period of the pigs exposed by the respiratory and tonsillar routes, caused by the bisecting of the trachea, necessitated the detection of virus in the blood at the earliest possible moment. The early sampling seemed to increase the efficiency of the system. Later infection through other body openings or susceptible membranes, resulting from contact with exudate from the exposed lung, probably would have resulted in delayed development of viremia beyond the sampling period in most instances.

SUMMARY AND CONCLUSIONS

The virus of hog cholera, carefully enclosed in double gelatin capsules to prevent contact of the virus and the pharyngeal tissues, proved incapable of producing hog cholera when liberated in the stomachs of susceptible swine. Animals exposed by the gastric route with the more concentrated samples of virus appeared to react more quickly and to develop more marked lesions on subsequent challenge than those exposed in the same manner to less concentrated virus, indicating a sensitizing effect of an apparently nonimmunizing antigen.

Successful infection through the tonsillar area was accomplished in pigs in which the trachea was severed to prevent possible respiratory infection. The virus was detected in the blood stream 24 hours postinoculation in pigs exposed by the tonsillar route.

Respiratory infection with hog cholera virus was successfully accomplished in pigs with tracheas that had been severed to prevent simultaneous exposure of the tonsillar area. The virus was detectable in the blood of pigs surviving for 24 hours or longer following exposure, but not in pigs which survived for 21 hours or less.

References

[1] Bodian, D.: Emerging Concept of Poliomyelitis Infection. Science, *122*, (1955): 105–108.

[2] Dunne, H. W., Reich, C. V., Hokanson, J. F., and Lindstrom, E. S.: Variations in Virus of Hog Cholera. A Study of Chronic Cases. Proc. Book, AVMA (1955): 148–153.

[3] Kernkamp, H. C. H.; University of Minnesota, St. Paul, Minn.: Personal communication, 1957.

[4] Parker, R. C.: Methods of Tissue Culture. 2nd ed. Paul B. Hoeber, Inc., New York, N.Y. (1950): 77.

[5] Schwarte, L. H., and Mathews, J.: Transmission of Hog Cholera Via the Respiratory Tract. North Am. Vet., *35*, (1954): 671–672.

[6] Shope, R. D.: Swine Influenza. In "Diseases of Swine," H. W. Dunne, ed. Iowa State College Press, Ames (1958): 81–98.

[7] Shope, R. E.: The Swine Lungworm as a Reservoir and Intermediate Host for Hog Cholera Virus. J. Exptl. Med., *107*, (1958): 609–622.

The Pathogenesis of Hog Cholera. II. The Virus Eclipse Phase and Sensitization of the Host

H. W. DUNNE, D.V.M., Ph.D., and A. J. LUEDKE, D.V.M.

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INTEREST in the pathogenesis of virus diseases has stimulated investigation into that stage of early virus infection in which infective virus cannot be detected in the infected system, or can be detected only in markedly decreased amounts. This phenomenon, commonly termed the virus eclipse stage, has been shown to occur with influenza virus *in vitro* in embryonated chicken eggs,[13] and in de-embryonated chicken eggs.[2,9] In summarizing the results of early workers with influenza virus, Burnett[1] states that "it is at least clear that there is a well marked eclipse phase lasting approximately three hours from the time of inoculation before any new virus particles, demonstrable either as hemagglutinin or as infective units, appear in the membranes."

Herpes virus grown in chicken embryo fibroblasts was shown to liberate the first virus about three and one half hours after inoculation and continued to release virus in "bursts" through seven hours or longer.[4] Vesicular stomatitis virus inoculated into guinea pig foot pads was not detectable at two hours in a 1:20 dilution of foot pad tissue, even though the original inoculum contained 10^6 infective doses.[11] Campbell,[3] in studying the virus of erythroleukemia in chickens, made intravenous inoculations and found that the virus disappeared in 30 minutes and did not reappear in the blood in detectable quantity until 18 hours. From previous work,[5] it appeared that the virus of hog cholera did not become detectable in the blood stream for several hours after inoculation into swine.

The experiments reported here were designed to investigate the existence of an eclipse phase in which no infective virus is detectable in the animal system, and some of the factors influencing this phase of virus multiplication.

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MATERIALS AND METHODS

Blood from experimental swine A510, A515, A942, and A943, infected with the "Sprankle" strain hog cholera virus,[7] was used in all experiments. One milliliter of this blood contained more than 1×10^6 but less than 1×10^7 virus infectious doses for swine. Except where indicated, the virus was diluted with a phosphate buffer, pH 7.4. Filtration through a Seitz sterilizing filter removed bacterial contaminants and particle aggregates.

Susceptible weanling pigs, 8 to 10 weeks old, were injected with 1 ml. of virus into the left jugular vein near its junction with the anterior vena cava. Blood samples were then taken from the right jugular vein of the injected animals at indicated intervals of 15 minutes to 48 hours.

In the experiments designed to determine the relative infectivity of blood and tissues, 3 pigs intravenously inoculated with 10^5 l.d. of A942–A943 hog cholera virus were killed at two, five, and eight hours. Reticuloendothelial tissues, including the pharyngeal, prefemoral, inguinal, and mesenteric lymph nodes, and the spleen were removed. Blood samples were taken at the time of killing.

Both suspensions of tissue and blood samples taken at the specific hours were divided into two portions. One half of the blood was diluted 1:1 with water. This diluted blood and one portion of the tissue suspension were frozen to lyse the cells and prevent further virus multiplication. The other half was refrigerated overnight at 4 C.

All samples were tested in swine within 24 hours after collection. Suspensions of these tissues were centrifuged to remove cellular debris and the supernatant fluid was tested for the presence of the virus. Since no completely satisfactory laboratory detection tests for hog cholera virus have been developed, the presence of virus in the samples was determined by injecting 1 ml. of the sample into hog cholera susceptible pigs. Samples diluted 1:1 with water for the purpose of lysing cells were injected in 2-ml. amounts.

All animals were placed in strict isolation during the course of the experiments. Animals surviving or showing no illness following injection were subsequently challenged with known infective hog cholera virus to determine susceptibility.

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EXPERIMENTAL PROCEDURES AND RESULTS

The usual incubation period of hog cholera in swine, based on temperature rise and leukopenia, is three to four days following injection with the virus. To insure recovery of the virus, the first eclipse studies were made over a 48-hour period. With one exception, the inoculations were made intravenously.

Experiments 1 and 2.—An identical inoculation of 1 ml. of a 1×10^{-1} dilution of blood virus A510–A515 (10^5 l.d.) was made into each of 2 pigs. Some of the sampling times were varied to provide a closer coverage of the first 24-hour sampling period. One milliliter of blood from each postinfection sample was injected into 1 or 2 susceptible swine (table 1). The virus was present at 15 minutes in the pig in experiment 2. It was not present in blood taken from either pig at 30 minutes nor was it demonstrated in the blood taken up to and including 13 hours after infection of the pig in experiment 1. All samples taken at 16 hours or later were positive. The challenge of animals given the noninfective samples was made four weeks after the initial inoculations and they were shown to be susceptible to the virus.

Experiment 3.—To determine the effect of a larger amount of the virus in the inoculum, a 30-lb. pig was inoculated with 1 ml. of undiluted blood virus (10^6 l.d.) (table 2). Blood taken at eight, 16, and 24 hours was positive for hog cholera virus. Samples were not taken earlier than eight hours, and it is possible that no eclipse phase may have been present.

Experiment 4.—Since it seemed that the time of appearance of the virus was directly related to the ratio between body size and the number of lethal doses in the inoculum, experiment 4 was designed with a similar virus of blood origin but a different lot (A942–A943), diluted 1:10 (10^5 l.d.), and with smaller pigs (22 lb.). In this experiment, the virus was not detectable at one, five, and eight hours, but was present at 12 hours (table 2). This pig had just been weaned, was two weeks younger, and weighed at least 8 lb. less than most pigs in these experiments. All these factors may have influenced the earlier appearance of the virus in the blood.

TABLE 1—Virus Eclipse in Experimentally Inoculated Swine

Experiment	Hour postinfection blood sample taken	Infectivity of sample in susceptible swine	Challenge of survivors
Experiment 1, diluted infected blood. Intravenous injection 1 cc. 1×10^{-1} dilution virus A510–A515. Pig weight, 30 lb.	30 min.	—	Died
	3 hr.	—	Died
	3 hr.	—	Died
	5 hr.	—	Died
	5 hr.	—	Died
	8 hr.	—	Died
	8 hr.	—	Died
	13 hr.	—	Died
	13 hr.	—	Died
	24 hr.	+(Died)	.....
	24 hr.	+(Died)	.....
	32 hr.	+(Died)	.....
	48 hr.	+(Died)	.....
Experiment 2, additional time periods. Same inoculation as above. Pig weight, 35 lb.	15 min.	+(Died)	.....
	30 min.	—	Died
	16 hr.	+(Died)	.....
	18 hr.	+(Died)	.....
	20 hr.	+(Died)	.....
	22 hr.	+(Died)	.....

Experiment 5.—Undiluted infected blood (10^6 l.d. of hog cholera virus) was inoculated into a 30-lb. susceptible pig (table 3). The animal was killed at five hours and the blood saved. Although this experiment was part of another study, the results were applicable to the work reported here. Of 9 pigs injected with the five-hour blood, 5 sickened with hog cholera; 4 of these died and 1 recovered. The incubation periods, which were lengthened, ranged from five to 13 days. All of the pigs which did not sicken were susceptible to hog cholera on subsequent challenge. Five hours was the earliest sampling period at which virus was detected following intravenous infection with a large amount of virus. This oc-

curred in one animal which was inoculated with 10^6 l.d. However, no blood samples earlier than five hours were taken from this animal to show that the virus was not present before this period. The possibility existed here also that, because of the larger inoculum, the virus was never entirely eliminated from the blood stream.

Experiment 6.—The time required for the appearance of virus in the blood following tonsillar infection was also determined within a broad range. Blood from an animal infected through the tonsillar area with a swab dampened with blood virus diluted 1×10^{-1} was negative when taken at one, eight, and 16 hours postinfection but positive at 24 hours (table 4). These findings compared favorably with earlier findings,[7] which indicated that pigs surviving a tracheal operation for 24 hours after tonsillar and respiratory exposures had detectable virus in their heart blood, whereas those that survived 20 hours or less did not.

Experiment 7.—To determine if virulent virus was present in the tissues of a pig infected eight hours or less, 3 pigs were inoculated with 1 ml. of virus A942–A943 and then killed at two, five, and eight hours, respectively. Samples of blood and lymph nodes taken at this time were divided into two equal portions as previously described. One portion of each was diluted 1:1 with sterile distilled water and frozen to lyse the cells, thus inhibiting further virus development. The other portion was refrigerated at 4 C. to retard, but not necessarily to inhibit completely, the viral growth. One ml. of the undiluted samples and 2 ml. of the diluted samples were inoculated subcutaneously into hog cholera-susceptible pigs the following day.

The results of this experiment compared favorably with the results of experiments 1, 2, and 4, in which virulent virus was not recovered before 12 and 16 hours. None of the injected animals developed typical hog cholera. Reactions were limited to various degrees of temperature rise (table 5). However, in table 5 a line is drawn between the 5-hour frozen and the 5-hour not frozen blood and tissues, thereby separating two groups equal in number but generally different in reaction. All samples above this line stimulated a relatively mild reaction in inoculated swine. All below this line caused reactions of a more pronounced nature. In the column showing temperatures taken on the sixth day, which was the first day temperatures of all animals were taken, all temperatures, with one exception, were 102.8 F. or lower in the upper half. The exception was the pig which had been inoculated with the five-hour frozen tissue sample and which attained a temperature of 103.4 F. In the lower half, all temperatures were 103.0 F. or higher. In like manner, the highest temperature recorded in the upper half in the column headed "Highest Temperature" was 103.8 F., whereas in the same column below the line, the temperatures ranged from 103.8 to 105.2. Leukocyte totals in all

TABLE 2—Effect of Virus Dilution on the Virus Eclipse Phase

Experiment	Hour postinfection blood sample taken	Infectivity of sample in susceptible swine	Challenge of survivors
Experiment 3, undiluted infected blood. Intravenous injection 1 cc. undiluted virus A510–A515. Pig weight, 30 lb.	8 hr.	+(Died)	.....
	16 hr.	+(Died)	.....
	24 hr.	+(Died)	.....
Experiment 4, diluted blood, smaller pigs. Intravenous injection 1 cc. of a 1×10^{-1} dilution virus A942–A943. Pig weight, 22 lb.	1 hr.	—	Died
	5 hr.	—	Died
	8 hr.	—	Died
	12 hr.	+(Died)	.....
	16 hr.	+(Died)	.....
	24 hr.	+(Died)	.....

TABLE 3—Variations in Response to 5-Hour Sample When Initial Virus Dose was Large

Pig No.	Incubation period (days)	Termination	Challenge
1	13	Died	.....
2	13	Died	.....
3	6	Died	.....
4	5	Died	.....
5	5	Recovered	Immune
6	.....	.....	Died
7	.....	.....	Died
8	.....	.....	Died
9	.....	.....	Died

Experiment 5, intravenous injection of identical samples of undiluted virus A510–A515. Pigs weighed 30 lb.

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animals were well within the normal range for swine.

A further difference indicated between the top half and the bottom half of table 5 is shown in the challenge area. High temperatures begin at 107.4 F. and reach a peak of 108.6 in the lower half. In the upper half, the peak is 107.4 F. and the low is 106.6. Only 1 of 6 animals listed in the upper half reached the peak. With 1 exception, all animals listed below the midline (table 1) died at 18 days or before. All pigs above the midline lived 18 days or longer. Also, the day on which the high temperature was reached, following challenge, was earlier in the group listed in the lower half of the table than in the group in the upper half. A statistical significance of less than 0.01 per cent was shown in the first two columns in the challenge area. The 1 odd pig (38) eliminated statistical significance in the last column. It is recognized, however, that the numbers are small for the establishment of a statistical analysis. In general, the group of samples in which the cells were lysed by hypotonicity and freezing produced a somewhat lesser response in injected animals than those which were placed in the 4 C. refrigerator overnight. This difference occurred also after challenge of the injected pigs with virulent virus four weeks later. There appeared to be little difference, however, between the response in animals injected with blood samples and the response in animals injected with corresponding tissue samples.

TABLE 4—The Effect of Tonsillar Infection on the Virus Eclipse Phase

Hour postinfection blood sample taken	Infectivity of sample in susceptible swine	Challenge of survivors
1	—	Died
8	—	Died
16	—	Died
24	+(Died)	.....

Experiment 6, tonsillar infection with virus A593 diluted 1 x 10^1. Pigs weighed 35 lb.

The lesions observed at necropsy were generally more intense in the animals inoculated with the unfrozen five-hour samples and all eight-hour samples than in the animals inoculated with all two-hour samples and the five-hour frozen samples. Although *Pasteurella multocida* was isolated from 3 of 6 animals in each of the two groups, pneumonia was most severe in the animals shown in the lower half of the table and these animals had the most severe septicemic lesions.

DISCUSSION

In an attempt to explain the reactions following challenge, it is perhaps well to consider some earlier observations by Dunne *et al.*[5,8] which indicated that pigs injected with components of hog cholera virus which were neither infective nor immunizing, gave evidence of sensitization on subsequent challenge with virulent hog cholera virus. This sensitivity reaction was characterized by higher temperatures, more severe hemorrhages and skin discolorations, shorter incubation periods, and shorter courses than were regularly seen in hog cholera-infected pigs.

The exact nature of this sensitization is not known. In general, the reaction of sensitized animals following challenge sim-

TABLE 5—The Eclipse Phase in Tissues and Blood Taken at Early Stages of Infection

	Source of incomplete virus			Reaction of pigs to incomplete virus			Challenge with virulent virus 4 wk. after test		
				Temperature (F)			Temperature		
Pig No.	Hour after infection	Tissue taken	Treatment of tissue	6th day	Highest	Day	High	Day	Course (day of death)
28	2	Blood	Frozen	101.6	102.6	9	107.0	10	18
29	2	l.n.	Frozen	101.8	103.4	9	106.6	9	22
30	2	Blood	Not frozen	102.8	103.0	9	107.4	7	23
31	2	l.n.	Not frozen	102.6	103.2	9	107.2	7	24
32	5	Blood	Frozen	102.4	103.4	11	107.0	7	24
33	5	l.n.	Frozen	103.4	103.8	11	107.0	9	18
34	5	Blood	Not frozen	103.4	105.0	9	108.0	6	15
35	5	l.n.	Not frozen	104.0	104.8	8	108.6	6	18
36	8	Blood	Frozen	103.0	103.8	12	107.8	3	12
37	8	l.n.	Frozen	103.8	105.2	13	107.8	6	16
38	8	Blood	Not frozen	105.0	105.0	6	108.0	6	31
39	8	l.n.	Not frozen	103.2	104.2	12	107.4	5	16

Line indicating a division of general reactions for discussion in text. l.n. = lymph node.

ulated the reaction one would expect from an inoculation with a virus of increased virulence. This suggests that, basically, the effectiveness of the natural defense mechanism of the animal against the invading virus has been decreased. The factors contributing to this lowered defense may be many. The properdin system may have been adversely affected, thus lowering the general resistance to disease. An indirect effect of an allergic phenomenon might result in the impairment of antibody-producing cells by histamine. Eosinophils are shown to be concentrated early in areas where the first lesions of hog cholera are seen.[10] These cells are reported to be active in the production of immunity.[12] The diversion of these cells from the area of infection by a generalized allergic phenomenon might decrease their effectiveness in altering the viral infection.

The response observed in sensitized animals following challenge is believed not to be an uncomplicated allergic reaction. The effects of a challenge of a sensitized animal cannot be observed before 24 to 48 hours. Even then, the first signs observed are not more than a temperature rise and a leukopenia. The more intensified lesions, not commonly seen in uncomplicated hog cholera, are observed at the peak of the disease or near its termination.

In the experiments reported in this paper, it appears that the early stage of hog cholera virus infection in the cell is characterized by the development of some components, probably incomplete virus particles, which are almost completely nontoxic and noninfectious. This development takes place in both blood and lymphatic tissue cells. This is emphasized by earlier work,[6,7] in which it was shown that the virus multiplies in leukocytes. The incomplete particles apparently do not have the antigenic structure which is peculiar to the complete virus particle. Antibodies developed against the incomplete particles are incapable of neutralizing virulent virus. The stimulus evoked by these particles, however, causes the host to respond to challenge with virulent virus by developing the severe acute disease described here in "sensitized" animals.

Since it was known that hog cholera could infect leukocytes *in vitro*,[6,8] it was only logical to assume that infection of these cells also would take place *in vivo*. It also seemed possible that virus infected leukocytes, when transferred intact and alive during the eclipse phase to a new susceptible host, would develop infective virus. Failure to transfer infection on the injection of blood samples taken during the eclipse phase suggested that the virus may have been picked up by the leukocytes within the first 15 minutes after the virus was injected into the blood, and removed from the blood stream within 30 minutes by the lymph nodes, spleen, and bone marrow.

The purpose of lysing and freezing blood and lymphatic tissue cells was to liberate the virus particles and to prevent further development. By this means, an accurate measurement of "completeness" of viral particles could be achieved. It was hoped that intact cells refrigerated at 4 C. overnight would continue to develop virus particles to completeness when injected into the new host. In the first instance, success was achieved in that virus development was immediately disrupted. In the case of refrigerated tissue, however, some virus development may have continued at a reduced rate, for the unfrozen cells caused a quicker and greater reaction to the initial inoculation into the pig as well as a greater degree of sensitization to subsequent challenge than did comparative frozen samples. However, any increase in virulence which may have been attained was not sufficient to kill or immunize the swine injected with any of the tissues or cells.

Pig 38 (table 5), which was inoculated with an eight-hour sample of unfrozen blood, reacted somewhat more severely to the initial inoculum than did pigs inoculated with comparable tissue samples and the frozen blood sample. Upon challenge, this pig reacted severely but lived significantly longer (31 days) than the pigs given the other eight-hour samples. This suggests that the virus particles in the eight-hour unfrozen blood sample may have progressed further toward completeness or more toward 1 m.l.d.* than those in any of the other eight-hour samples, and that the antibodies produced were more nearly specific for the complete virus particle than those produced by injections of the other

* It is generally considered that hog cholera produces an all or none response, in that 1 m.i.d. is equal to 1 m.l.d.

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eight-hour samples. The increased specificity of antibodies would therefore mean more increased resistance to the virus. Although the specificity obviously was not enough to prevent death, it could have caused the lengthened course of disease.

SUMMARY

Virulent hog cholera blood virus injected intravenously into susceptible swine was present in the blood stream at 15 minutes postinfection but not at 30 minutes. It was detected again at 12 hours when the inoculating dose did not exceed 10^5 l.d. When the dose was 10^6 l.d., the virus was detected as early as five hours postinoculation, and there may not have been a period in which virus was completely absent from the blood stream.

Blood cells and lymphatic tissue cells refrigerated or frozen at two hours, five hours, and eight hours postinfection proved noninfectious in swine. Animals injected with five-hour unfrozen, eight-hour unfrozen, and eight-hour frozen blood and tissue cells showed somewhat greater reactions than those injected with five-hour frozen and two-hour frozen and unfrozen blood and tissue. Those which showed the more pronounced reactions subsequently appeared "sensitized" to challenge with virulent virus. Freezing of hypotonic suspensions of cells appeared to stop viral multiplication. There appeared to be a little evidence of virus multiplication at five hours. Refrigeration appeared to retard but not completely stop viral multiplication.

The phenomenon of "sensitization" was discussed and theories for its occurrence were presented.

References

[1] Burnet, F. M.: Principles of Animal Virology. Academic Press Inc., New York, N.Y. (1955): 142.

[2] Burnet, F. M., Lind, P. E., and Stevens, K. M.: Production of Incomplete Influenza Virus in the Deembryonated Egg. Austral. J. Exptl. Biol. & M. Sci., *33*, (1955): 127–141.

[3] Campbell, J. G.: Observation on the "Eclipse Phase" in a Virus-Associated Erythroleukemia of the Chicken. Brit. J. Cancer, *8*, (1954): 737–742.

[4] Dulbecco R., and Vogt, M.: One-Step Growth Curve of Western Equine Encephalomyelitis Virus on Chicken Embryo Cells Grown *in Vitro* and Analysis of Virus Yields from Single Cells. J. Exptl. Med., *99*, (1954): 183–199.

[5] Dunne, H. W., Hokanson, J. F., and Luedke, A. J.: The Pathogenesis of Hog Cholera. I. Route of Entrance into the Body. Am. J. Vet. Res., (1959): In press.

[6] Dunne, H. W., Luedke, A. J., and Hokanson, J. F.: The Growth of Animal Leukocytes and Their Use in the Cultivation of Animal Viruses. Am. J. Vet. Res., *19* (July, 1958): 706–711.

[7] Dunne, H. W., Luedke, A. J., Reich, C. V., and Hokanson, J. F.: The *in Vitro* Growth of Hog Cholera Virus in Cells of Peripheral Blood. Am. J. Vet Res., *18*, (July, 1957): 502–507.

[8] Dunne, H. W., Reich, C. V., Hokanson, J. F., and Lindstrom, E. S.: Variations in the Virus of Hog Cholera. A Study of Chronic Cases. Proc. Book, AVMA (1955): 148–153.

[9] Hanig, M., and Bernkopf, H.: The Sedimentable Components of Influenza Virus Propagated in Deembryonated Eggs. J. Immunol., *65*, (1950): 585–590.

[10] Luedke, A. J., and Dunne, H. W.: The Pathogenesis of Hog Cholera. III. Eosinophic Infiltration of Lymphatic Tissues. In preparation for publication.

[11] Sabin, A. B., and Olitsky, P. K.: Influence of Host Factors on Neuroinvasiveness of Vesicular Stomatitis I and II. J. Exptl. Med., *66*, (1937): 15–57.

[12] Spiers, R. S.: Advances in the Knowledge of the Eosinophil in Relation to Antibody Formation. New York Acad. Sci. *73*, (1958): 283–306.

[13] Von Magnus, P.: Propagation of the PR8 Strain of Influenza A Virus in Chick Embryos. II. The Formation of "Incomplete" Virus Following Inoculation of Large Doses of Seed Virus. Acta. path. et microbiol. scandinav., *29*, (1951): 278. Cited by Burnet, F. M.: Principles of Animal Virology. Academic Press Inc., New York. (1955): 134–135.

Isolation of a Cytopathogenic Agent from Swine with Enteritis

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THE STUDY of viral diarrhea in swine has interested American workers for many years. In 1946, Doyle and Hutchings [2] first described gastroenteritis in suckling pigs that was transmissible to other litters. Diarrhea was reproduced by feeding a filtrate prepared from a portion of infected enteric tract, complete with its contents.

Transmissible gastroenteritis (TGE) is clinically characterized by diarrhea, vomiting, loss of weight, and a high mortality among the litters. Adult swine become infected but seldom succumb. Other epizootics have been reported by Feenstra *et al.*[3] and Whitehair *et al.*[7] In all cases, the disease showed similar signs, and the responsible agent was demonstrated and transmitted with the same technique as described above. As far as we know, no cytopathogenic effect (CPE) has been demonstrated in tissue culture for the agent of TGE. However, Haelterman [4] informed us that the agent multiplies in swine kidney tissue culture, but without CPE.

This is a report of our experience with an epizootic of diarrhea in suckling pigs which occurred in Rome, Italy, during 1956. The clinical features of this disease and the properties of the agent recovered differ in significant respects from that of TGE.

MATERIALS AND METHODS

Twenty suckling pigs, in four litters, which had shown diarrhea over a four-day period, were studied. They were located on the farm of a commercial hog raiser. The rest of the herd, including the mother sows, were without clinical signs of infection. Rectal swabs were prepared for inoculation into tissue culture by making a suspension in 2 ml. of Hank's balanced salt solution. Monkey and swine kidneys used for primary isolation were prepared for tissue culture according to the method of Dulbecco, as modified by Youngner.[8]

RESULTS

A cytopathogenic agent was recovered from 1 suckling pig in a litter of 6, all of which had diarrhea; some also evidenced anorexia and cachexia but, unlike TGE, no vomiting occurred. Five of the 6 pigs recovered; 1 died on the eleventh day of illness, when it was 15 days old. It was from this fatal case that the agent was recovered in monkey kidney cells. A definite cytopathogenic effect, indistinguishable from that of polio virus, was observed.

The virus was passed in swine kidney tissue culture, and it behaved in the same way. At the third level of passage in monkey and swine kidney, the agent showed a titer of $10^{5.4}$ 50 per cent tissue culture dose ($t.c.d._{50}$) per 0.2 ml. The $t.c.d._{50}$ is the dose of virus required to produce a cytopathogenic effect in half of the tubes inoculated, as calculated by the Reed and Muench method.[6] No CPE resulted, however, from passing the virus in rabbit kidney, human kidney, or HeLa cells. The virus did not agglutinate chicken erythrocytes at room temperature. Attempts to infect newborn pigs by intraperitoneal or oral routes were unsuccessful. Similar attempts with suckling and adult mice, using various routes, were likewise negative. In a single experiment with 2 monkeys infected orally (2 ml. per monkey of infected, undiluted tissue culture fluid), diarrhea resulted in both animals after two days. The same swine virus persisted in the stools of both monkeys for 12 days. Acute phase serum and serum taken after 30 days were tested for neutralizing antibodies. Antibodies were tested in rhesus or cynomolgus monkey kidney cell cultures using 100 $t.c.d._{50}$ of virus (as measured by tissue culture titration) mixed with equal parts of twofold serial dilutions of either serum. Following one hour of incubation of the mixture at room temperature, 0.3-ml. aliquots of the serum-virus mixture were added to the tubes of monkey kidney tissue culture. They were observed after incubation at 37 C. on the fourth and eighth day. Inhibition of cytopathogenesis was the criterion of the neutralization power of the serums. One monkey showed a rise in homologous antibody (from 0 to 1:128). The other monkey had no titer in either serum.

In an attempt to evaluate the prevalence of this virus in the swine population, adult

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pigs were swabbed rectally at the central slaughterhouse in Rome, where animals from the numerous farms of the surrounding Lazio region (an area the size of New Jersey) are collected. No cytopathogenic agent was recovered.

Neutralization tests were performed as described above and were uniformly negative. However, the serum of the sow which had a litter with diarrhea from which virus was not recovered neutralized 100 $t.c.d._{50}$ of the recovered virus at a final dilution of 1:10. Unfortunately, no serum could be obtained from the suckling with the fatal infection.

Serums from 15 normal pigs were without neutralizing activity against our virus, as was human *gamma* globulin and monkey anti-polio serum. Antiserums against hog influenza, transmissible gastroenteritis, porcine vesicular stomatitis and exanthema, and canine distemper were similarly lacking in neutralizing effect in tissue culture.* A porcine serum prepared against atrophic rhinitis of pigs, which was of questionable activity against that agent, was also without neutralizing effect on this swine diarrhea virus. We did find that undiluted antiserum from a pig which had recovered from hog cholera (Italian) neutralized our swine diarrhea virus.

Human serums from healthy Italian children were tested against our swine virus. Neutralization of 100 $t.c.d._{50}$ of the swine virus was effected by six of the healthy children's serums at a final dilution of 1:10. Antiserums prepared against the swine diarrhea virus in rabbits were tested against enteric cytopathogenic human orphan (ECHO) viruses 1 through 6, using the method described. This serum did not neutralize 100 $t.c.d._{50}$ of the ECHO viruses at a final dilution of 1:10.

One lot of rabbit serum prepared against ECHO virus 8 neutralized the swine virus at a titer of 1:128. A second lot prepared elsewhere had no activity. The reaction is not symmetrical, as anti-swine virus serum is incapable of neutralizing ECHO 8 virus. It is too early in the study of these agents to speculate whether there is more than a suggestion of relationship of the swine diarrhea virus to ECHO viruses.

SUMMARY AND CONCLUSIONS

A cytopathogenic virus was recovered from an epizootic of diarrhea in suckling pigs, which differed significantly from the agent of transmissible gastroenteritis. It also differed from the swine orphan virus recovered by Beran *et al.*,[1] in that it was not, like his virus, recovered from normal adult or suckling pigs. It had no antigenic relationship to his virus. Furthermore, no antibodies were detected against our virus in normal hogs, whereas Beran found a high incidence of neutralizing antibodies in the serum and colostrum of normal hogs.

The agent reported in this study might, for the time being, be classified as an enteric cytopathogenic swine orphan virus (ECSO), as suggested by Hsiung and Melnick.[5] It grows well in monkey and swine kidney tissue cultures. Diarrhea was not reproduced with this virus in suckling pigs, but in 1 monkey the production of diarrhea, with a rise in homologous antibody, was successful. This suggests that the virus is potentially pathogenic for at least one host and may have been the causative agent of the epizootic studied.

ADDENDUM

Since this paper was submitted we have been able to test this swine virus against two pig serums, namely DF 5 and ODL 235, sent to us by Dr. P. H. Lamont, School of Veterinary Medicine, Cambridge, England. These two serums belong to pigs from which a virus (V-13) was isolated. One hundred $t.c.d._{50}$ of our swine virus were neutralized by these two serums as a 1:50 final dilution.

* The authors thank Dr. Beran, who made these serums available.

References

[1] Beran, G. W., Werder, A. A., and Wenner, H. A.: Enteroviruses of Swine. I. Their Recognition, Identification, and Distribution in a Swineherd. Am. J. Vet. Res., *19*, (July, 1958): 545–553.

[2] Doyle, L. P., and Hutchings, L. M.: A Transmissible Gastroenteritis in Pigs. J.A.V.M.A., *108*, (April, 1946): 257–259.

[3] Feenstra, E. S., Thorp, F., Gray, M. L., and McMillen, W. N.: Transmissible Gastroenteritis of Baby Pigs. J.A.V.M.A., *113*, (Dec., 1948): 573–575.

[4] Haelterman, E. O.; Purdue University, Lafayette, Ind.: Personal communication, 1956.

[5] Hsiung, G. D., and Melnick, J. L.: Orphan Viruses of Man and Animals. Ann. N.Y. Acad. Sci., *70*, (1958): 342–361.

[6] Reed, L. J., and Muench, H.: A Simple Method of Estimating Fifty Percent Endpoints. Am. J. Hyg., *27*, (1938): 493.

[7] Whitehair, C. K., Grunner, R. M., Phillips, P. H., Bohstedt, G., and McNutt, S. H.: Gastroenteritis in Pigs. Cornell Vet., *38*, (1948): 23–38.

[8] Youngner, J. S.: Monolayer Tissue Cultures. I. Preparations and Standardization of Suspensions of Trypsin-Dispersed Monkey Kidney Cells. Proc. Soc. Exptl. Biol. & Med., *85*, (1954): 202–205.

Studies on Myoclonia Congenita. IV. The Segmental Reflex in Normal and Affected Pigs

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PREVIOUS PAPERS [5,15,16] have dealt with several aspects of myoclonia congenita, a disease of newborn pigs characterized by a tremor present at birth. This paper presents the results of an electrophysiological study comparing the segmental response in control, affected, and recovered pigs. The spinal cord segmental reflex, since first described by Eccles and Pritchard,[3] has become a useful instrument in the study of neuronal activity.

Contributions to the understanding of the anatomic basis and functional significance of the segmental reflex have been made by a number of authors.[1,2,4,6-8,10-14] To the authors' knowledge there have been no reports in the literature describing electrophysiological studies of spinal cord in pigs.

MATERIAL AND METHODS

Three groups of young pigs were used in this study. Group 1, composed of 8 pigs having no history of tremor, was used as a control group. Group 2 consisted of 8 pigs showing typical tremor. Group 3 contained 7 pigs which had recovered from the disease and showed no clinical evidence of tremor. The pigs were obtained either from litters farrowed at the College of Veterinary Medicine or from outlying farms in the state as described in the paper on field studies.[15]

The anesthetic (Dial-urethane*) was injected initially via an ear vein and subsequently by means of a cannula in the external jugular vein. The trachea was cannulated to permit artificial respiration, if needed. A laminectomy was performed by removal of the spinous processes and arches of the last two lumbar and first two sacral vertebrae. The exposed dura was slit mid-dorsally to expose the dorsal and ventral roots of the spinal cord. The region was then flooded with warm (39 C.) mineral oil. The arrangement of the stimulating and recording electrodes is shown (fig. 1).

Both stimulating and recording electrodes were constructed from silver wire. The stimuli were delivered either by a thyratron multichannel stimulator or a Grass S4B square wave stimulator.† The reflexly elicited action potentials were recorded using an R-C coupled amplifier, a dual-beam cathode ray oscilliscope, and a recording camera (fig. 2).

In general, the response was tested in each animal at stimulation frequencies of both one per second and one per four seconds. The stimulus intensity was set at a low level and gradually increased until a response was observed on the screen of the oscilloscope. The stimulus intensity was then increased a small amount following each sweep of the oscilloscope beam until the maximum response was obtained.

All measurements of the segmental reflexes were made from the image produced by projecting the film, using a photographic enlarger. Whenever practicable, the degree of enlargement was adjusted so that a time interval of 1 millisecond (msec.) was represented by a distance of 10 mm. Latencies were measured to the nearest tenth of a millisecond.

Data were accumulated from the last lumbar, first and second sacral segments. Since they make up the largest portion of the study, only experiments performed on the first sacral (S1) segments will be discussed fully.

RESULTS

Group 1, Normal Pigs.—The segmental reflex obtained by stimulating the dorsal root of S1 and recording from the ventral root of the same segment (fig. 3) was similar to that obtained in the cat by Lloyd.[9] The response consists, in general, of two main components. Following the stimulus artifact by a latency of 1.4 msec. was a synchronous spike which has been called the monosynaptic response. Following the monosynaptic reponse closely was a wave of longer duration referred to as the multisynaptic response. One notable and reasonably constant characteristic observed in the multisynaptic response of pigs was the appearance of two well-defined spikes, one or both of which often exceeded the mono-

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* Dial-urethane, supplied by Ciba Pharmaceutical Products, Summit, N.J.

† Manufactured by Grass Instrument Co., Quincy, Mass.

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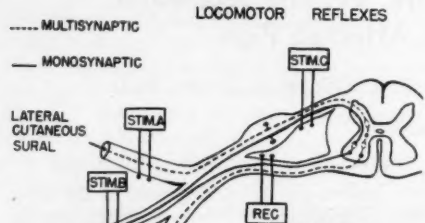

Fig. 1—The stimulus is applied to the cut dorsal root (stim. C) and response (C) is recorded (rec.) from the cut ventral root. The multisynaptic (A) and monosynaptic (B) response can be obtained separately by stimulating at sites such as stimulus A and stimulus B, respectively.

synaptic response in terms of size. These spikes do not appear as constantly in the segmental responses of the cat. These two spikes will be referred to as the second and third waves, respectively. These waves were especially prominent when the stimulus intensity was fairly high, but in all instances the multisynaptic response appeared to have the same or lower threshold than the monosynaptic response (fig. 4A).

The monosynaptic reflex response was obtained in all of the animals tested, but showed considerable fluctuation. It was not a constant feature of the segmental reflex response throughout each experiment, and in most instances appeared to be depressed rather rapidly as the experiment progressed. The unfacilitated monosynaptic response appeared to be by far the most sensitive portion of the segmental reflex response with respect to condition of the animal. Any respiratory difficulties were almost immediately reflected in a reduction of the height of monosynaptic response. When an overdose of the anesthetic was used to kill the pig at the end of an experiment, the monosynaptic response disappeared long before the rest of the ventral root response.

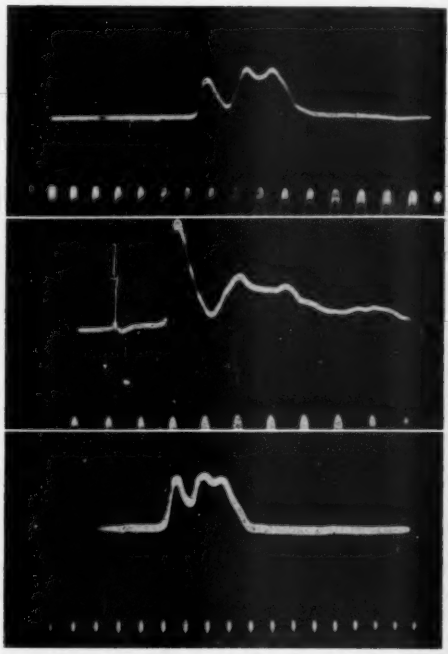

Fig. 3—Segmental reflex (first sacral segment) response in normal (top), affected (center), and recovered (bottom) pigs. Notice the relatively greater monosynaptic response in the tremor-affected pig. Time is in milliseconds.

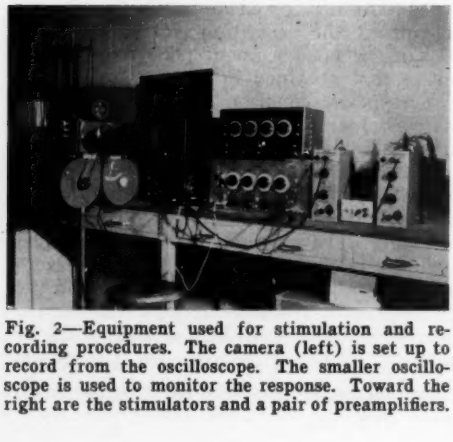

Fig. 2—Equipment used for stimulation and recording procedures. The camera (left) is set up to record from the oscilloscope. The smaller oscilloscope is used to monitor the response. Toward the right are the stimulators and a pair of preamplifiers.

The maximum latencies of monosynaptic response were seen at low intensities of stimulation (fig. 4). The range of recorded minimum latencies for the 8 pigs in group 1 was 1.7 to 1.0 msec. The mean value for minimum latency was 1.36 msec. (table 1).

Duration of the monosynaptic spike varied between 0.5 and 1.3 msec., and the mean value for duration was 0.97 msec. (table 1).

Maximum height of the monosynaptic response was also measured as a part of this series. These values have no significance in themselves, since constancy of response amplification could not be maintained from one experiment to another. However, these maximum values for the monosynaptic response are herein expressed in terms of a ratio of the monosynaptic maximum to the second wave maximum. For the normal pigs, this ratio varied between a minimum of 0.02 and a maximum of 2.84. The mean value for ratio of monosynaptic response maximum (amplitude) to second wave response maximum in 8 pigs was 0.77 for the S1 segment (table 1).

These values for monosynaptic response latency, duration, and amplitude ratio were obtained with a stimulus frequency of one shock per four seconds. Reducing the interval between stimuli to one second resulted in no marked changes in these average measurements. The values obtained at the two different frequencies are listed (table 4).

The characteristics of the segmental reflex response were noticed to differ considerably when tested with other stimulating frequencies. To illustrate this, the results obtained in 1 pig will be described. With respect to the monosynaptic response, the latency at a stimulus frequency of one per ten seconds was 1.4 msec. The latency then decreased slightly and maintained a minimum until a stimulus frequency of 20 shocks per second was reached. The latency values then began to increase with frequency until a maximum of 1.8 msec. was reached, when stimulating with 150 shocks per second. At this latter frequency, the ventral root response was almost completely inhibited. At the various stimulation frequencies tested, the duration of the monosynaptic response appeared to fluctuate more or less randomly between 1.0 and 1.3 msec. The comparative absolute values of spike height for first, second, and third waves at the various frequencies are graphically illustrated (fig. 5). Variation of latency with frequency of stimulation is shown (fig. 6).

The maximum latencies of multisynaptic response were also seen at low intensities of stimulation. The range of recorded latencies for the second wave response in these pigs was 4.7 to 2.3 msec. (table 1). The mean value for minimum latency was 2.56 msec. The mean value for duration of second and third wave was 2.60 msec., but it must be emphasized that this value is not as accurate as that given for the monosynaptic spike. The reason for this is the

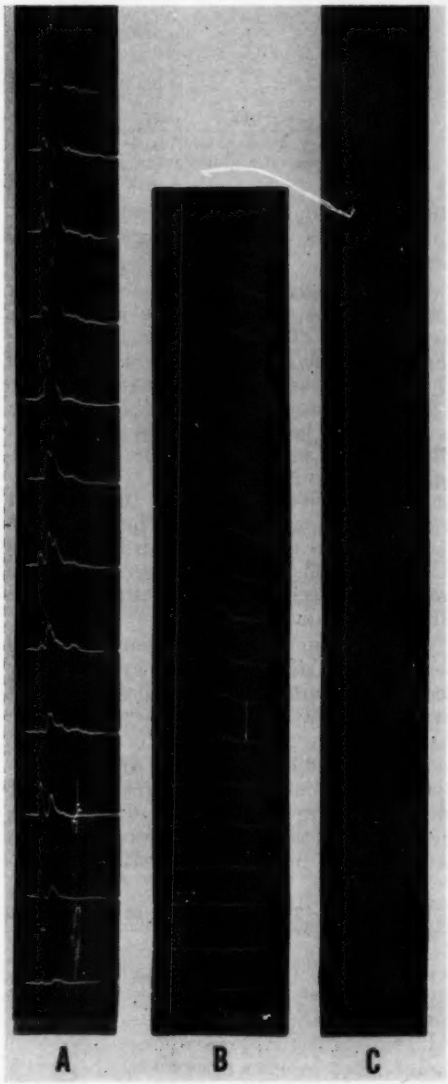

Fig. 4—Segmental reflex (first sacral segment) response to increasing (from bottom to top) strength of stimulus in (A) normal, (B) affected, and (C) recovered pigs. Notice the relative magnitude of the monosynaptic response in the three groups and its lack of lability in the tremor-affected pig. Time is in milliseconds.

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TABLE 1—Data on the Segmental Reflex Response in Normal Pigs

Pig No.	Maximum latency 1st wave	Minimum latency 1st wave	Duration 1st wave	Max. ht. 1st wave / 2nd wave	Maximum latency 2nd wave	Minimum latency 2nd wave	1st peak to 2nd	2nd peak to 3rd	Duration 2nd & 3rd waves	Threshold of 1st & 2nd waves
64	2.0	1.5	1.3	0.40	4.7	3.3	1.4	0.8	2.9	Same
65	2.3	1.7	0.5	0.07	3.4	2.4	1.2	1.0	2.5	2 lower
66	1.5	1.4	0.7	0.02	3.0	2.4	1.4	0.9	2.3	2 lower
68	1.5	1.0	1.2	2.84	3.2	2.5	1.3	0.6	2.2	Same
69	1.8	1.5	0.9	1.07	3.9	2.4	1.4	....*	1.9	2 lower
72	1.9	1.3	1.1	0.94	3.4	2.3	1.2	1.1	2.8	Same
88	2.0	1.3	1.0	0.22	3.9	2.5	1.5	1.0	2.8	Same
89	2.1	1.2	1.1	0.60	3.5	2.7	1.9	1.0	3.5	2 lower
Av.	1.89	1.36	0.97	0.77	3.62	2.56	1.41	0.91	2.60	.....

All latency, duration, and peak-to-peak values are in milliseconds.
* Not measurable in this animal.

difficulty encountered in sharply delineating the third wave with respect to later activity.

A new parameter of segmental reflex characteristics was made use of in these experiments. This parameter is the time lapse between first and second spike and that between second and third spike. The relative constancy of these values for each animal and between animals suggests a physiological significance. The values obtained are indicated (table 1). The mean value for time lapse between the first and second wave was 1.41 msec. The mean time lapse between second and third wave was 0.91 msec. The individual values of the latter measurement varied comparatively little from animal to animal.

The characteristics of the multisynaptic response at a stimulus frequency of one per second are quite similar to those observed at stimulus intervals of four seconds (table 4).

Some of the characteristics of the multisynaptic response at various frequencies of stimulation are shown (fig. 5, 6). A fairly gradual increase of second wave response latency was noted as the stimulus frequency was increased. The first peak to second peak values showed a rather irregular although definite increase with frequency of stimulation. No well-defined third wave could be observed at stimulus frequencies higher than three per second. However, some activity still remained in the time region formerly occupied by this third wave. In terms of amplitude, the second wave response did not show the sharp increase in the 8 to 15 stimuli-per-second region where the monosynaptic response amplitude was seen to rise sharply. Graphs of response variation with frequency of stimulation are needed from many more animals before any assessment of their significance can be considered.

Group 2, Pigs with Tremor.—The recorded response characteristics of this group are summarized (table 2). The degree of tremor involvement indicated is based on visual observation and is only intended as a guide to more accurate interpretation of the quantitative data.

Mean values for the various ventral root reflex characteristics (table 4) and a record of ventral root responses to a graded series of dorsal root stimuli in a group 2

TABLE 2—Data on Segmental Reflex Response in Pigs with Tremor

Pig No.	Maximum latency 1st wave	Minimum latency 1st wave	Duration 1st wave	Max. ht. 1st wave / 2nd wave	Maximum latency 2nd wave	Minimum latency 2nd wave	1st peak to 2nd	2nd peak to 3rd	Duration 2nd & 3rd waves	Threshold of 1st & 2nd waves
67*	1.8	1.1	1.1	1.49	3.5	2.3	1.2	0.9	3.2	Same
70†	1.4	1.2	1.4	2.35	3.4	2.5	1.8	0.9	4.3	Same
71†	2.1	1.5	1.5	7.33	4.0	3.3	2.4	0.7	2.9	1 lower
78*	2.0	1.6	0.8	1.06	3.5	2.7	1.2	0.6	2.5	Same
79**	2.0	2.0	1.2	5.75	4.0	4.0	3.0	0.8	4.0	Same
81*	1.4	1.2	1.1	3.37	2.9	2.6	1.3	....‡	....	1 lower
84**	1.6	1.3	2.0	4.39	4.8	3.9	2.9	(1.7)§	5.2	1 lower
87**	1.7	1.0	1.0	1.85	3.0	2.2	1.2	....	3.0	Same
Av.	1.75	1.36	1.26	3.45	3.64	2.94	1.87	0.78	3.59	.....

All latency, duration, and peak-to-peak values are in milliseconds.
* slight tremor; † moderate tremor; ** severe tremor; ‡ quantity not measurable; § this value was questionable, so it was not included in the average value.

TABLE 3—Data on Segmental Reflex Response in Recovered Pigs

Pig No.	Maximum latency 1st wave	Minimum latency 1st wave	Duration 1st wave	Max. ht. 1st wave / 2nd wave	Maximum latency 2nd wave	Minimum latency 2nd wave	1st peak to 2nd	2nd peak to 3rd	Duration 2nd & 3rd waves	Threshold of 1st & 2nd waves
57	1.9	1.3	1.1	1.54	3.0	2.7	1.4	....*	2.3	Same
58	2.0	1.8	1.5	1.22	3.4	....	1.4	1.0	1.8	2 much lower
59	1.8	1.2	1.0	1.45	3.3	2.4	1.6	....	1.0	2 much lower
62	1.7	1.3	1.0	0.36	3.2	2.8	1.9	....	3.3	Same
73	2.5	1.8	0.8	0.10	3.4	2.6	1.3	....	2.7	2 much lower
74	2.1	1.4	1.2	1.58	4.0	2.6	1.3	0.8	2.6	1 lower
82	1.8	1.3	0.8	0.51	3.6	2.3	1.2	1.0	2.5	2 lower
Av.	1.97	1.44	1.06	0.97	3.41	2.57	1.44	0.93	2.31	.....

All latency, duration, and peak-to-peak values are in milliseconds.
* Quantity was not measurable.

pig (fig. 4) are shown. Figure 3 includes an enlarged photograph of a maximal segmental reflex response.

Group 3, Pigs Recovered from Tremor.— The recorded response characteristics are summarized (table 3). None of these animals showed any visible indications of tremor before being anesthetized.

The mean values for ventral root reflex response (table 4) and a record of ventral root response to a graded series of dorsal root stimuli in a group 3 pig (fig. 4) are shown. Figure 3 includes an enlarged photograph of a maximal segmental reflex response.

TABLE 4—Average Group Values for Characteristics of the Segmental Reflex

Group	Maximum latency 1st wave	Minimum latency 1st wave	Duration 1st wave	Max. 1st / Max. 2nd	Maximum latency 2nd wave	Minimum latency 2nd wave	1st peak to 2nd	2nd peak to 3rd	Duration 2nd & 3rd waves	Frequency
1	1.89	1.36	0.97	0.77	3.62	2.56	1.41	0.91	2.60	1/4
1	1.81	1.43	0.96	0.73	3.57	2.53	1.40	1.10	2.90	1/sec.
2	1.75	1.36	1.26	3.45	3.64	2.94	1.87	0.78	3.59	1/4
2	1.68	1.36	1.20	2.69	3.55	2.93	1.70	0.85	3.49	1/sec.
3	1.97	1.44	1.06	0.97	3.41	2.57	1.44	0.93	2.31	1/4

All undesignated values are in milliseconds, except those in the fifth column which are ratios of amplitude.

DISCUSSION

Since no information is available in the literature on the electrical neurophysiology of the porcine spinal cord, it is not possible to directly compare these results with those of others. Judging from Lloyd's illustrations,[9] the general configuration of the ventral root reflex response from the first sacral segment of the spinal cord of the cat is quite similar to that in the pig. This is especially apparent at high intensities of dorsal root stimulation, where Lloyd's records show the height of the multisynaptic response exceeding that of the monosynaptic. However, the early parts of the multisynaptic response in the pig appear to be somewhat more synchronous than in the cat.

Lloyd's [9] records of reflex activity in the cat at low intensities of stimulation show monosynaptic and multisynaptic firing appearing at the same threshold. This is also characteristic of the ventral root response in several of the control (group 1) pigs. In others, the second wave appeared at a lower intensity of stimulation than the first wave (table 1). However, these differences in threshold of monosynaptic and multisynaptic activity were not marked.

Minimum latencies for monosynaptic response varied remarkably little among the three groups of pigs. Considering that the time values were measured to the nearest tenth of a millisecond, these averages (table 4) could be considered essentially identical.

It is apparent (table 4) that the first wave response is far greater in pigs with tremor (group 2) than in either control or recovered (group 3) pigs. Also, the first wave appeared at the same or lower intensities of stimulation than did any part of the multisynaptic response in all pigs with tremor. Taking into account the duration and comparative magnitude of monosynaptic response, it seems evident that the two-neuron-arc pathways in pigs with tremor

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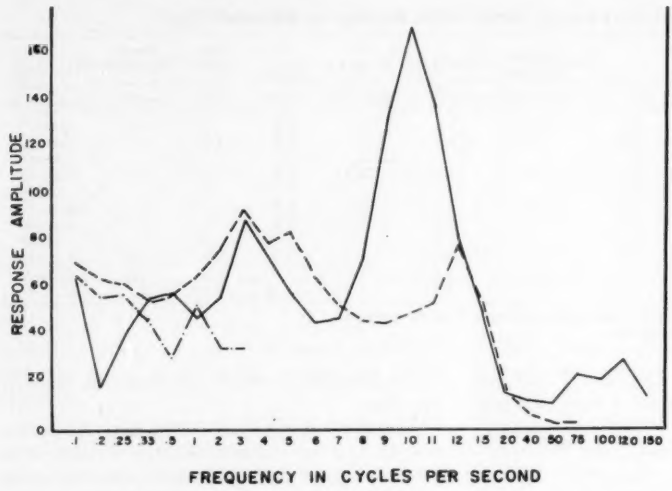

Fig. 5—Comparative amplitude of first (———), second (– – – – –), and third (— — — —) wave responses as a function of frequency of stimulation in a normal pig. Notice the sharp rise in monosynaptic response amplitude in the 8- to 15-stimuli-per-second region.

may be brought into action much more readily than the multisynaptic pathways. Reference to minimum latency values for second wave and first peak to second peak values (table 4) shows that the multisynaptic response is delayed in pigs with tremor. Therefore, the multisynaptic response can be seen to be both delayed and depressed in these affected animals.

Based on the above findings, there appears to exist a hyperexcitable monosynaptic and hypoexcitable multisynaptic response in pigs with tremor. However, the question of whether the reduced multisynaptic activity is secondary to the excessive monosynaptic response remains unanswered.

It is recognized that supraspinal influences probably play a part in shaping the character of the segmental reflex response obtained in all of the pigs. These influences are being investigated.

The characteristics of reflex responses in recovered pigs do not appear to differ greatly from those of normal pigs. The only notable difference is in the ratio of maximum first to maximum second wave (table 4), and this difference is not large.

SUMMARY

A study has been conducted on segmental reflex response in 23 pigs. These included normal pigs, pigs showing tremor, and pigs recovered from tremor. The results were analyzed with respect to a variety of characteristics, and the segmental reflex response in pigs with tremor was found to differ markedly from that in normal pigs.

Segmental reflex characteristics in re-

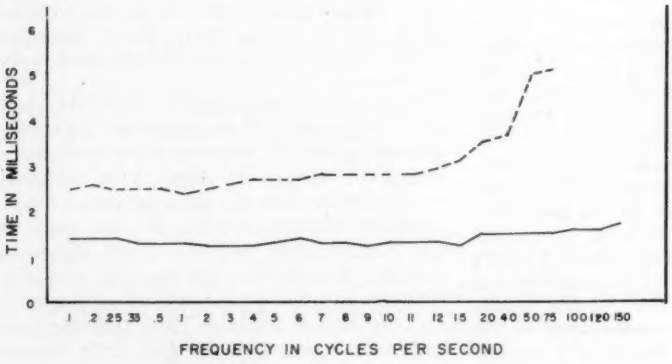

Fig. 6—Variation of latency of segmental reflex response as a function of stimulus frequency in a normal pig. The multisynaptic response is represented by the broken line, the monosynaptic response by the solid line. Change of stimulus frequency had little effect on latency of monosynaptic response.

covered pigs did not differ notably from those in normal pigs, except possibly with respect to maximum amplitude ratios of first to second wave. The nature of the segmental reflex response in pigs with tremor suggests increased excitability via two-neuron-arc pathways and decreased excitability via multineuronal pathways.

References

[1] Alvord, E. C., Jr., and Fuortes, M. G. F.: Reflex Activity of Extensor Motor Units Following Muscular Afferent Excitation. J. Physiol., *122*, (1953): 302–321.

[2] Brooks, C. McC., and Koizumi, K.: A Comparison of the Monosynaptic and Polysynaptic Reflex Response of the Spinal Cord Under a Variety of Influences. In "Ciba Foundation Symposium on the Spinal Cord." Little, Brown & Co., Boston (1953): 63–77.

[3] Eccles, J. C., and Pritchard, J. J.: The Action Potential of Motoneurones. J. Physiol., *89*, (1937): 43–45.

[4] Granit, R.: Receptors and Sensory Perception. Yale University Press, New Haven, Conn. (1955): 191–276.

[5] Hanson, L. J., Stromberg, M. W., Kitchell, R. L., and Kernkamp, H. C. H.: Studies on Myoclonia Congenita. II. Gross and Microscopic Pathology. Am. J. Vet. Res., *19*, (April, 1958): 383–387.

[6] Kühne, W.: Über die Endigung der Nerven in den Muskeln. Virchows Arch. path. Anat., *27*, (1863): 508–533.

[7] Kühne, W.: Die Muskelspindeln. Virchows Arch. path. Anat., *28*, (1863): 528–538.

[8] Lloyd, D. P. C.: Reflex Action in Relation to Pattern and Peripheral Source of Afferent Stimulation. J. Neurophysiol., *6*, (1943): 111–119.

[9] Lloyd, D. P. C.: Conduction and Synaptic Transmission of the Reflex Response to Stretch in Spinal Cats. J. Neurophysiol., *6*, (1943): 317–326.

[10] Lorente de No, R.: Limits of Variation of the Synaptic Delay of Motoneurones. J. Neurophysiol., *1*, (1938): 187–194.

[11] Renshaw, B.: Activity in the Simplest Spinal Reflex Pathways. J. Neurophysiol., *3*, (1940): 373–387.

[12] Ruffini, A.: Observations on Sensory Nerve-Endings in Voluntary Muscles. Brain, *20*, (1897): 368–374.

[13] Ruffini, A.: On the Minute Anatomy of Neuro-Muscular Spindles of the Cat and on Their Physiological Significance. J. Physiol., *23*, (1898): 190–208.

[14] Sherrington, C. S.: On the Anatomical Constitution of Nerves of Skeletal Muscles: With Remarks on Recurrent Fibers in the Ventral Spinal Nerve-Root. J. Physiol., *17*, (1894): 211–258.

[15] Stromberg, M. W., and Kitchell, R. L.: Studies on Myoclonia Congenita. I. Review of Literature and Field Investigations. Am. J. Vet. Res., *19*, (April, 1958): 377–382.

[16] Stromberg, M. W.: Studies on Myoclonia Congenita. III. Drugs and Other Factors Affecting Severity of Tremor in Pigs. Am. J. Vet. Res., *20*, (March, 1959): 319–323.

[17] Szentágothai, J.: Anatomical Consideration of Monosynaptic Reflex Arcs. J. Neurophysiol., *11*, (1948): 445–454.

Correction—Sulfaquinoxaline in Dairy Cattle

In the article, "Studies on the Oral Administration of Sulfaquinoxaline in Dairy Cattle—Pharmacology and Pathology," by G. E. Brightenback, F. V. Washko, and O. H. Siegmund, in the October, 1958 issue of the *American Journal of Veterinary Research* (p. 794), the last paragraph of the Discussion, beginning with the sentence starting on line six, should read "*Unpublished reports*[28] *attest to the efficacy of this drug. . .* "

Reference 28, not included in the article and erroneously given (*Am. J. Vet. Res.*, May, 1959: 429), should read "[28] Personal communications: Frank Vigue, D.V.M., Springdale, Maine, Oct. 31, 1951; Ross Brown, D.V.M., University of Kentucky, Lexington, 1950; D. F. Eveleth, D.V.M., North Dakota Agricultural Experiment Station, Fargo, Jan. 22, 1954; and M. P. Chapman, D.V.M., Sherwood, Ore., March 22, 1955."

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Experimental Infection of Swine with *Brucella Melitensis* (Mediterranean Type)

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THE ANIMAL RESERVOIR of *Brucella melitensis* in the United States until 1946 was believed to be restricted to goats and primarily to a limited geographic area in the Southwest, where infections have been known since 1911.[6] As recently as 1945,[18] an outbreak of goat-borne brucellosis, involving a number of human cases, occurred in Colorado. The infected goats were destroyed and, at present, *Br. melitensis* infection in goats is believed to be of low incidence and to occur almost exclusively in states along the Mexican border.

Prior to 1946, *Brucella suis* was considered to be the only cause of naturally occurring swine brucellosis in this country. In December of 1943,[12] *Br. melitensis* was isolated from two packing plant workers in Iowa. With the exception of a Mexican, who became ill shortly after arriving in the United States, this was the first report of *Br. melitensis* infection in Iowa. By June of 1945, 23 additional humans with cases of *Br. melitensis* infection had been reported in the state. The source of exposure appeared to be swine; however, it was not until 1946 [2] that *Br. melitensis* was isolated from naturally infected swine in the same area.

Between December, 1948, and April, 1949, McCullough *et al.*[15, 16] confirmed the occurrence of *Br. melitensis* infection in swine by isolating this species from 11 hogs sampled at random in a Chicago packing plant. In May of 1949, Hutchings *et al.*[10] isolated *Br. melitensis* from a herd of swine in southern Indiana. These animals exhibited marked clinical evidence of brucellosis, indistinguishable from that due to *Br. suis*. Damon [4] isolated *Br. melitensis* from a hog in Indiana in the same year. From epidemiological evidence, infection with this species has been present in swine in Minnesota since 1945.[18]

During the past ten years, there has been an apparent increase in both swine and human brucellosis due to this species of Brucella. Geographically, these infections appear to be largely confined to the Midwest and, in man, have occurred principally in packing plant employees working in the pork department or in farmers having contact with hogs.

The pathogenesis of this type of swine brucellosis was studied by Hoerlein [8] in 1952. Employing a field strain of *Br. melitensis* recently isolated from swine on an Iowa farm where brucellosis in man had occurred, it was demonstrated that hogs may be infected experimentally by one of three routes: intravenous, conjunctival sac, and intravaginal. Contact infection was also demonstrated by isolation of *Br. melitensis* from uninoculated controls. It was concluded that the pathogenesis of *Br. melitensis* infection in swine is similar to that due to *Br. suis*; the strain used, however, "seemed moderate in virulence as compared with some strains of *Br. suis*."

Hoerlein provided us with the strain of *Br. melitensis* used in the previously mentioned study. On examination, we found this strain to possess characteristics similar to those of all strains of *Br. melitensis* which we (N. B. McC. and G. A. B.) have isolated from swine and from packing plant workers; *i.e.*, growth in the presence of both thionine and basic fuchsin, the production of only a trace or no hydrogen sulfide, and high urease activity. For purposes of convenience and differentiation, we shall refer to such strains as American type *Br. melitensis*.

The origin of *Br. melitensis* (American type) has never been satisfactorily explained. Further, no adequate study has been made of the infectivity for swine of *Br. melitensis* of the goat-borne variety. Huddleson,[9] in 1944, stated: "Melitensis is pathogenic for the hog. While I was in Malta in 1938 I saw evidence of its pathogenicity. A considerable amount of left-over unpasteurized goat's milk was fed to a group of pregnant sows. All of them aborted. Melitensis was recovered from the fetuses. For some reason this observation was never reported in the literature."

Under routine typing procedures, the Mediterranean or classic type of *Br. melitensis* is not distinguished from the American type. However, within recent years tests for urease activity have been employed by an increasing number of investigators in the characterization and typing of Brucella cultures. Cultures which type as *Br. melitensis*, on the basis of H_2S production and dye sensitivity, fall into two distinct categories in regard to their urease activity, with the American type producing significantly greater amounts of this enzyme. Because of their epidemiological history, strains of *Br. melitensis* which are character-

From the Department of Health, Education, and Welfare, Public Health Service, National Institutes of Health, National Institute of Allergy and Infectious Diseases, Laboratory of Bacterial Diseases, Bethesda, Md. (Beal and McCullough); and the Department of Veterinary Science, Purdue University, Lafayette, Ind. (Taylor, Claflin, and Hutchings).

ized by relatively low urease activity are conveniently referred to as the classic or Mediterranean type.

It would be of considerable epidemiological significance if strains of *Br. melitensis* (goat-borne, Mediterranean, or classic type), as a result of their maintenance and growth in swine tissue, became altered so as to be indistinguishable from the American type. The present investigation was undertaken to determine: (1) the infectivity for swine of representative strains of the Mediterranean type of *Br. melitensis* and, in the event infection was established, the characteristics thereof; and (2) the influence of residency in tissues of swine on characteristics which distinguish the classic type of *Br. melitensis* from the American type.

Materials and Methods

Cultures.—Four strains of *Br. melitensis* were used. Strain 1 was isolated from a man in Saudi Arabia;* strain 2 was isolated in our laboratory (N. B. McC. and G. A. B.) from a patient who had recently arrived in this country from southern Italy; strain 3 was isolated at Beltsville, Md., from a kid born to an infected goat which had been shipped from a herd in New Mexico;† strain 4 was isolated from a patient at Michigan State University, but was originally derived from a patient in Malta.

The cultural and typing characteristics of all four strains were typical of the Mediterranean type of *Br. melitensis.* All grew on trypticase soy agar containing either thionine or basic fuchsin; the test results for H_2S production were negative, or only trace amounts were produced when grown on liver infusion agar or trypticase soy agar slants; more than one hour was required to obtain a positive urease test using the method of Bauer as recommended by the Joint FAO/WHO Expert Committee on Brucellosis;[11] and all were agglutinated to high titer in antiserums prepared by infecting rabbits with smooth Brucella organisms and were not agglutinated by normal serum.

On the basis of reciprocal absorption tests, they were serologically distinguishable from *Br. suis* and *Brucella abortus.* Their pathogenicity for guinea pigs, determined prior to their use in this study, was of a high order, infections being produced by the intraperitoneal inoculation of as few as 10 viable organisms. Each of the four strains possessed individual characteristics which enabled us to distinguish one from the others.

Swine.—A total of 24 Hampshire gilts and barrows were used. They were from farm herds with no history of brucellosis and were 2 to 3 months old when the experimental studies were started. They were kept on grounds at Purdue University and observed for a two-week period before use. Blood samples drawn immediately prior to experimental exposure to *Br. melitensis* were negative to the agglutination test for brucellosis, and attempts to culture Brucella from them were unsuccessful.

Preparation of Inoculums.—The cultures of *Br. melitensis,* representing single colony selections, were grown for 24 hours at 37 C. on trypticase soy agar slants. The growth was suspended in 0.85 per cent NaCl and adjusted turbidimetrically to provide the desired number of organisms in a final volume of 5 ml. In all instances, plate counts were made to establish the actual number of viable Brucella therein.

Bacteriological Studies.—All Brucella cultures recovered during the course of the study were subjected to bacteriological examination to determine if there had been any alteration in character from that possessed by the four strains at the time the study was initiated. Among the characteristics studied were: colony morphology, antigenic behavior, sensitivity to thionine and basic fuchsin, hydrogen sulfide production, and urease activity. Finally, for each of the four strains, the Brucella culture representing its maximum residency in swine tissue was tested for its pathogenicity for guinea pigs.

Determination of Urease Activity.—Bauer's method for determining urease activity was modified slightly to afford more quantitative results. Each isolate was tested twice. The first test was performed on unselected growth and the second after single colony selection. The 48-hour growth on trypticase soy agar slants was suspended in saline solution and, after photoelectric measurement, a volume of the suspension containing an estimated 10 billion viable Brucella organisms was centrifuged. The supernatant fluid was discarded and to the sedimented cells was added 1 ml. of urease test solution. The packed cells were then uniformly resuspended and incubated at 37 C. A reading for color change from yellow to red was made at 15-minute intervals for the first two hours and at 30-minute intervals thereafter for a minimum of four hours. A control suspension, similarly prepared, representing the strain prior to pig exposure, was always tested in parallel. A second control, confirming dependability of the test conditions, consisted of a suspension of *Br. melitensis* (American type).

Determination of Infectivity for Guinea Pigs.—Forty male guinea pigs, N.I.H. strain, weighing 350 to 450 Gm., were inoculated in groups of 10 animals each with graded dilutions of standardized suspensions of each strain of *Br. melitensis.* The suspensions were prepared from cells representing the second successive 24-hour growth from single, selected colonies. The size of the inoculum was approximated turbidimetrically and the number of viable cells was determined by surface plating to trypticase soy agar. The inoculums, in a volume of 0.5 ml., were given intraperitoneally. After 3(

* Supplied by Dr. I. F. Huddleson, Michigan State University, East Lansing.
† Supplied by Dr. C. M. Manthei, Beltsville, Md.

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TABLE 1—Results of Oral Exposure of Swine to Brucella Melitensis

Pig No.	Exposure data: No. organisms (millions)	Exposure data: Strain (No.)	Brucella agglutinin titer: Time after exposure (days) 7	14	21	28	35	42	56	63	71	77	83	90	PE day killed
1	7,100	1	N*	1:25	N	N	N	1:25	N	N	.....	.....	.....	.....	65†
2	142		N	1:25	1:100	1:50	1:25	1:25	1:25	1:25	.....	.....	.....	.....	65
3	2,425	2	N	1:25	1:25	1:25	1:25	1:50	1:25	1:25	N	1:25	N	N	98
4	48		N	N	N	1:50	N	1:25	1:25	N	N	N	N	N	98
5	1,400	3	N	N	1:25	1:50	1:50	1:25	1:25	1:50	1:50	1:50	1:25	1:50	98
6	27		N	N	N	N	N	N	N	N	N	N	N	N	99
7	5,600	4	N	N	1:50	1:25	1:25	N	1:25	N	N	N	N	N	99
8	106		N	N	N	1:50	1:50	1:25	1:50	1:25	1:25	1:25	1:25	1:25	99

* N = negative for Brucella agglutinins (blood cultures taken at time blood was drawn for agglutinin titer were negative for Brucella). † *Brucella melitensis* was recovered from mandibular lymph node (Brucella was not recovered from the other animals at necropsy).

days, the guinea pigs were tested for agglutinins, killed, and the following tissues cultured for Brucella: spleen, liver, inguinal and axillary lymph nodes, and testes. The criterion of infection was the recovery of Brucella at necropsy.

First Experiment—Exposure of Swine to *Brucella Melitensis* Via the Oral Route.—Two shoats were used for each of the four strains of *Br. melitensis* (table 1). The bacterial suspensions were sprayed into the throat of each animal with a syringe. Blood samples were taken at weekly intervals after exposure, and the serum was tested for Brucella agglutinins using the BAI standard plate antigen; the dilutions tested were 1:25, 1:50, 1:100, 1:200, and 1:400. Also at weekly intervals, 5 ml. of blood was obtained under aseptic conditions from the anterior vena cava, put into culture bottles containing approximately 40 ml. of tryptose broth, and incubated at 37 C. for two weeks. Subcultures from broth were made at five to seven days by withdrawing 1 ml. of the blood-broth mixture and using this to inoculate a plate of tryptose agar. Second subcultures were made at the end of the two-week period. Two pigs (those given *Br. melitensis* strain 1) were killed 65 days after exposure. The remaining 6 (exposed to strains 2–4) were killed approximately 100 days after exposure.

At necropsy, the following tissues were cultured for Brucella: lymph nodes (popliteal, prefemoral, superficial inguinal, internal iliac, gastrohepatic, mesenteric, mandibular, and cervical); kidneys, liver, spleen, heart, lung, uterus, and ovaries. Culturing was done by smearing the cut surfaces of the tissues on individual plates of tryptose agar. The plates were then incubated at 37 C. in a normal atmosphere for one week. These culture techniques and isolations were performed at Purdue University.

Second Experiment—Exposure of Swine to *Brucella Melitensis* Via the Subcutaneous and Intravenous Routes.—Two shoats were used for each of the four strains of *Br. melitensis* and were inoculated subcutaneously in the left groin. At the same time, 2 additional shoats per strain were given similar doses of each of the organisms via the anterior vena cava. The cultures and method of preparation for inoculation were the same as those used in the group exposed orally.

A schedule of weekly blood samples for culture and agglutinin response was followed for a total of 16 weeks and then at biweekly intervals until termination of the experiment. Pigs inoculated subcutaneously were killed five months after exposure and those inoculated intravenously, approximately nine months after exposure. The

TABLE 2—Summary of Isolations of Brucella Melitensis from Experimentally Infected Pigs

Pig No.	Strain No.	Exposure route* and inoculum	Bacteremia: Times positive/ times cultured	Duration of bacteremia (days)	Period of observation (days)	Necropsy results: Titer	Necropsy results: Culture
Gilt 1	1	Oral: 7.1×10^9	0/8	0	65	Negative	*Br. melitensis* No. 1
Gilt 14	2	Subcutaneous: 62.0×10^9	1/18	15	154	1:50	Negative
		Intravenous:					
Gilt 9	1	102.0×10^9	13/26	134	265	1:200	*Br. melitensis* No. 1
Barrow 11		5.1×10^9	7/26	59	265	1:50	Negative
Gilt 13	2	62.0×10^9	6/26	89	265	1:50	Negative
Barrow 15		3.1×10^9	7/26	89	265	1:50	*Br. melitensis* No. 2
Gilt 17	3	64.0×10^9	8/26	111	265	1:400	*Br. melitensis* No. 3
Barrow 19		3.2×10^9	4/26	51	265	1:100	Negative
Gilt 21	4	72.0×10^9	7/26	119	265	1:400	Negative
Barrow 23		3.6×10^9	6/26	97	265	1:25	Negative

* For each exposure route 8 pigs were used.

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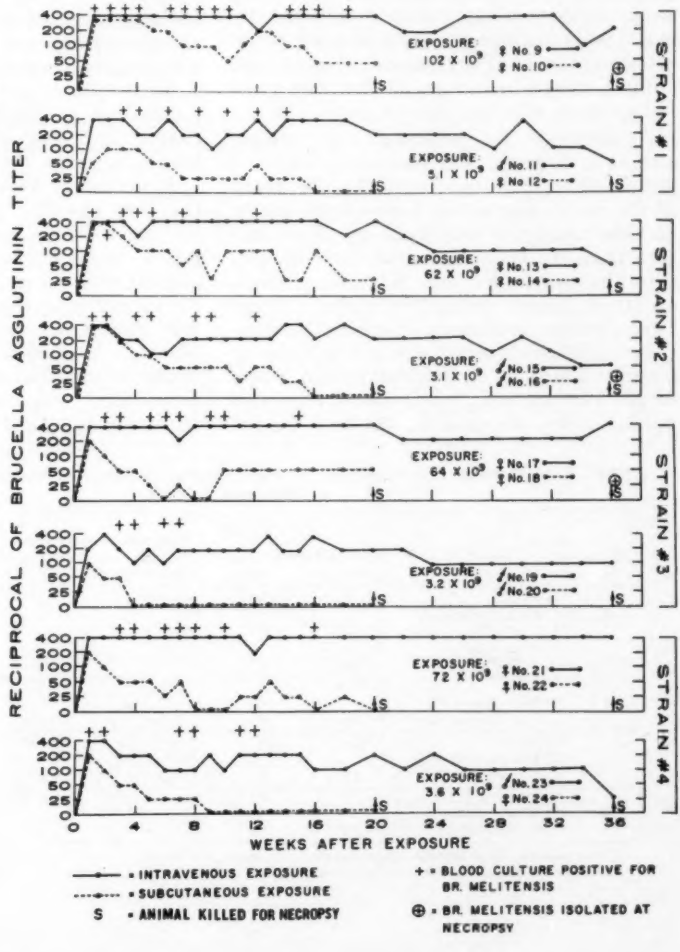
organs and tissues cultured for Brucella at necropsy corresponded to those of the group exposed orally. Necropsy and culture of tissues of the pigs exposed subcutaneously were carried out at Purdue University. Of the group exposed intravenously, necropsy was performed at Purdue University and the tissues were transported under refrigeration to the National Institutes of Health (N.I.H.) for culture. The techniques employed at N.I.H. were similar to those used at Purdue, with the exception that trypticase soy agar was used as the culture medium and, since this was the terminal experiment, a more rigorous effort was made to recover Brucella, including in some instances the preparation of pooled lymph node homogenates and their culture both by direct plating and by guinea pig inoculation.

RESULTS

Infectivity for Swine.—The exposure dose, agglutinin response, and culture results of pigs exposed orally are shown (table 1). Corresponding data for pigs exposed by subcutaneous and intravenous inoculation are shown (graph 1). A composite table (table 2) summarizes results obtained on animals from which Brucella was recovered at any time after exposure by any of the routes employed.

In only 1 pig of the 8 exposed orally was infection established, as determined by isolation of *Br. melitensis* 65 days after exposure. The degree of infection was slight; bacteremia was not demonstrated and the agglutinin response was negligible. Based on agglutinin response, 6 of the remaining 7 pigs in this group showed evidence of exposure to Brucella. The single exception was the animal given the smallest exposure dose.



Graph 1—Comparison of agglutinin titers and culture results of pigs exposed subcutaneously and intravenously to four strains of Brucella melitensis.

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Of the 8 shoats exposed subcutaneously, bacteremia was demonstrated in only 1 animal, 14 days after exposure to an estimated 62 billion organisms of strain 2. All of the pigs in this group, however, did develop Brucella agglutinins to a significant titer, and of the 4 pigs given the heaviest inoculum, 3 had demonstrable agglutinins when killed approximately 5 months after exposure. Brucella was not recovered from any of these animals at necropsy, and no lesions were found.

Infection was established in all of the 8 shoats exposed intravenously. The range of duration of *Br. melitensis* bacteremia was from seven to 18 weeks, and the number of positive hemocultures from each pig varied from a minimum of four to a maximum of 13. Although no positive blood cultures were obtained after the eighteenth week, significant agglutinin titers were demonstrable until they were killed, 36 weeks after exposure. They showed no marked clinical signs of disease and the only gross lesions were enlarged lymph nodes.

At necropsy, *Br. melitensis* representing strains 1, 2, and 3 was recovered from the lymph nodes of 3 pigs (table 3). Recovery of *Br. melitensis* strain 1 was from gilt 9, and the isolations were made by direct culture from the bronchial, iliac, cervical, and mandibular lymph nodes. *Brucella melitensis* strain 2 was isolated by direct culture from the bronchial node of barrow 15. *Brucella melitensis* strain 3 was not isolated by direct culture of individual lymph nodes of gilt 17; however, lymph nodes from this animal were trimmed of fat and pooled and a homogenate was prepared using the Waring blendor. From the homogenate, *Br. melitensis* was isolated by plating to trypticase soy agar. Recovery was not made by guinea pig inoculation of aliquots of this homogenate. A similar technique was used for the tissues of barrow 19 (also exposed to strain 3) and for the tissues of both pigs inoculated with strain 4 (gilt 21 and barrow 23), but Brucella was not recovered.

Characteristics of Brucella Isolated from Experimentally Infected Swine.—A total of 80 isolations of Brucella were made from 10 pigs which were exposed orally, subcutaneously, or intravenously to one of four strains of *Br. melitensis*. A total of 59 isolations represented recovery by hemoculture, and the remaining 21 were isolated from lymph nodes at necropsy.

Each isolation was treated as an individual culture and examined in parallel with cultures representing the four parent strains. Characteristics studied included colonial morphology, serology, dye sensitivity, hydrogen sulfide production, and urease activity.

A period of two years elapsed between the time the first pigs were exposed and the last isolation was made. Cultures were typed promptly as they were isolated; consequently, a number of batches of mediums and test reagents were used to obtain the data necessary for typing all isolates. Variations in test results which might be attributed to differences in mediums and test reagents were kept to a minimum by strict adherence to standard

TABLE 3—Cultural Characteristics of Four Strains of Brucella Melitensis, Before and After Residence in Swine

Strain No.	Source of isolate	Days resident in swine	Colony type	Growth on dye media		Urease activity time* (hours)	Test for H_2S production (5 days)
				Thionine 1:80,000	Basic fuchsin 1:80,000		
1	Stock	0	S	++++	++	5–6	N–T
	Mandibular node of gilt 1	65	S	++++	++	5–6	N–T
	Iliac node of gilt 9	265	S	++++	++	5–6	N–T
2	Stock	0	S	+++	++++	3	N–T
	Bronchial node of barrow 15	265	S	+++	++++	3	N–T
3	Stock	0	S	++	+++	3	N–T
	Lymph node pool from gilt 17	265	S	++	+++	3	N–T
4	Stock	0	SI	+++	+++	3	N–T
	Blood culture from gilt 21	119	SI	+++	+++	3	N–T

S = smooth, SI = smooth intermediate, N = none, T = trace.
* Time in hours for positive test using Bauer's method.

test procedures; *i.e.*, age and physiological state of culture, type of basic medium, the use of a single lot of certified dyes, and age of dye plates. The variable which was most difficult to control was in the results of tests for hydrogen sulfide production as new batches of liver infusion agar were prepared. However, in all instances in which a culture produced blackening of the lead acetate strip, the same degree of blackening was produced by the parent culture, which had been tested in parallel. Had this control not been included, the apparent change in ability to produce H_2S may have been falsely attributed to enzymatic change consequent to exposure to the tissues of swine.

Culture and typing characteristics of each of the four strains, before and after maximum residency in swine, are listed (table 3). Although the table includes only results of tests made after maximum residency in swine for each strain, all cultures isolated were tested and in no instance was there alteration even in individual strain peculiarities.

The infectivity for guinea pigs of isolates representing the longest period of residency in swine for each strain is given (table 4). In the case of strain 1, the pathogenicity of two isolates was measured; one from a pig exposed orally and one from a pig exposed intravenously. Included in the table are data obtained for each parent strain prior to use in the experimental study.

Discussion

The interhost transmissibility of the species of Brucella has not received attention commensurate with its importance. While it is true that for each of the recognized species of Brucella there is a primary and principal animal host, careful investigations have revealed that animals other than those principally concerned may be naturally infected with the other species, and thus may serve as sources of infection not

TABLE 4—The Effect of Hog Passage on the Infectivity for Guinea Pigs of Four Strains of Brucella Melitensis

Strain No.	Source of isolate	Days resident in swine	Inoculum (viable count)	Guinea pig infectivity (No. infected/ No. inoculated)
	Stock (before exposure to swine)	0	1,500	9/9
			150	7/10
			15	6/9
			1	0/10
1	Mandibular node of gilt 1	65	1,500	10/10
			150	9/10
			15	4/10
			1	1/10
	Iliac node of gilt 9	265	2,700	10/10
			270	10/10
			27	4/10
			2	3/10
	Stock (before exposure to swine)	0	850	10/10
			85	10/10
			8	9/9
2	Bronchial node of barrow 15	265	3,300	9/10
			330	10/10
			33	7/10
			3	0/10
	Stock (before exposure to swine)	0	4,000	10/10
			400	8/10
			40	7/10
			4	4/10
3	Lymph node pool from gilt 17	265	3,200	10/10
			320	10/10
			32	7/10
			3	4/10
	Stock (before exposure to swine)	0	1,700	9/10
			170	8/10
			17	4/10
			1	0/10
4	Blood culture from gilt 21	119	1,600	7/10
			160	4/10
			16	3/10
			1	0/10

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only for other animals but also for man.

Several workers have reported what they consider to be instances of species transformation of Brucella which they have attributed to residency in animal tissues other than that of the natural host. A comprehensive critique of this thesis has been presented recently by McCullough and Beal.[14] In such instances, the alleged transformations have been to that of the species most commonly known to produce natural infection in the animal under observation. Thus, the most plausible explanation for isolation of a species of Brucella other than that inoculated (the basis for reports of *in vivo* species transformation) is either latent or subsequent infection.

In the present study, four strains of *Br. melitensis* (Mediterranean type) were maintained as long as 265 days in swine, and multiple and repeated isolations throughout the interval yielded cultures which, upon careful examination, showed no alteration in character over that possessed before such exposure. It may be argued that passage through a series of swine may have resulted in alteration of the Mediterranean type of *Br. melitensis* to the American type; however, in the instances in which species alterations were reported, the reputed transformations occurred without such passage and after comparatively brief periods of residency in the aberrant host. Repeated passages were made in the studies of Cameron and Meyer,[3] Gilman *et al.*,[7] and Doyle,[5] and no alteration in species characteristics was observed.

It is not known how long brucellosis due to the American type of *Br. melitensis* has existed in the United States. Although human cases, some of which occurred in 1943, were first reported in 1945,[1] Huddleson, in August of 1933, had typed a culture isolated by Feldman from a bone lesion of a hog in Minnesota and classed it as *Br. melitensis* on the basis of H_2S and dye reactions. Subsequently, this organism[17] was shown to possess high urease activity such as is characteristic of the American type.

The geographic distribution of the American type of *Br. melitensis* is not accurately known. However, the organism has been isolated either from swine, from packing plant workers, or farmers exposed to swine in Colorado, Illinois, Indiana, Iowa, Massachusetts, and Minnesota. It may be presumed that if every culture of Brucella isolated were carefully typed and classified (without prejudice to its source), the American type of *Br. melitensis* would be found to occur in many more areas than have yet been reported.

From data presented here, there is no evidence that the Mediterranean type of *Br. melitensis* becomes converted to the American type as a result of residency in the tissues of swine. In addition, there is no evidence that the enzyme urease determines the pathogenicity of Brucella for swine. The strain which appeared to have the greatest infectivity for pigs was culture 1, which was least active as a producer of urease.

The results obtained in the present study appear to be entirely similar to those which Hoerlein[8] observed with the American type. While our observations were limited to a small number of subjects, the disease was similar to that reported by many workers for experimental infection with *Br. suis*.

SUMMARY

Shoats were exposed to four strains of *Brucella melitensis* (Mediterranean type) by spraying suspensions of organisms into the throat, and by subcutaneous and intravenous injection. Low agglutinin titers developed in 7 of the 8 pigs exposed orally, but bacteremia was not demonstrated and in only 1 pig was *Br. melitensis* isolated at necropsy 65 days after exposure.

Of the 8 shoats exposed subcutaneously, bacteremia was demonstrated in only 1 animal, 14 days after exposure, but significant agglutinin titers developed in all 8 pigs. Of those given the largest inoculum, 3 had demonstrable agglutinin titers at time of necropsy five months after exposure, but Brucella was not recovered from any of the group at that time.

Infection was established in all of the 8 shoats exposed intravenously, with bacteremia ranging in duration from seven to 18 weeks. Significant agglutinin titers were still present when the animals were killed, 36 weeks after exposure. At necropsy, *Br. melitensis* was recovered from the lymph nodes of 3 pigs.

No alteration in species characteristics or in individual strain peculiarities was

observed at any time, nor was infectivity for the guinea pig changed by prolonged residency in swine.

References

[1] Borts, I. H.: Some Observations Regarding the Epidemiology, Spread and Diagnosis of Brucellosis. J. Kansas M. Soc., *46*, (1945): 399–405.

[2] Borts, I. H., McNutt, S. H., and Jordan, C. F.: *Brucella Melitensis* Isolated from Swine Tissues in Iowa. J.Am.M.A., *130*, (1946): 966.

[3] Cameron, H. S., and Meyer, M. E.: An Attempt to Alter the Characteristics of *Brucella Abortus* by Serial Passage Through Swine. Cornell Vet., *42*, (1952): 42–45.

[4] Damon, S. R., and Scruggs, J. H.: Recovery of *Brucella Melitensis* from the Hog. Pub. Health Rep., *65*, (1950): 374.

[5] Doyle, T. M.: *Brucella Abortus* Infection of Goats. J. Comp. Path. & Therap., *52*, (1939): 89–115.

[6] Gentry, E. R., and Ferenbaugh, T. L.: Endemic Malta (Mediterranean) Fever in Texas with Isolation of the *Micrococcus Melitensis* from Two Patients. J.Am. M.A., *57*, (1911): 889–890.

[7] Gilman, H. A., Milks, C. H., and Birch, R. R.: Passage of Bovine Brucella Through Swine. J. Infect. Dis., *54*, (1934): 171–174.

[8] Hoerlein, A. B.: Studies in Swine Brucellosis. I. The Pathogenesis of Artificial *Brucella Melitensis* Infection. Am. J. Vet. Res., *13*, (Jan., 1952): 67–73.

[9] Huddleson, I. F.: Personal communication (*see* Jordan and Borts [12]).

[10] Hutchings, L. M., McCullough, N. B., Donham, C. R., Eisele, C. W., and Bunnell, D. E.: The Viability of *Brucella Melitensis* in Naturally Infected Cured Hams. Pub. Health Rep., *66*, (1951): 1402–1408.

[11] Joint FAO/WHO Expert Committee on Brucellosis: Second Report, Geneva, 1953, World Health Organ., Tech. Rep. Ser., *67*, (1953): 23.

[12] Jordan, C. F., and Borts, I. H.: Occurrence of *Brucella Melitensis* in Iowa. J.Am.M.A., *130*, (1946): 72–75.

[13] Kabler, P., Bauer, H., and Nelson, C. B.: Human *Brucella Melitensis* Infections in Minnesota with Hogs as the Probable Source. J. Lab. & Clin. Med., *32*, (1947): 854–856.

[14] McCullough, N. B., and Beal, G. A.: The Biological Stability of the Genus Brucella. Bull. World Health Organ., *19*, (1958): 725–737.

[15] McCullough, N. B., Eisele, C. W., and Pavelchek, E.: Isolation of *Brucella Abortus* from Hogs. Pub. Health Rep., *64*, (1949): 537–538.

[16] McCullough, N. B., Eisele, C. W., and Pavelchek, E.: Survey of Brucellosis in Slaughtered Hogs. Pub. Health Rep., *66*, (1951): 205–208.

[17] Sanders, E., and Warner, J.: Urease and Catalase Activities of *Brucella Melitensis* from Different Geographical Regions. Am. J. Vet. Res., *14*, (July, 1953): 388–391.

[18] Stiles, G. W.: Brucellosis in Goats. Recovery of *Brucella Melitensis* from Cheese Manufactured from Unpasteurized Goats Milk. Rocky Mountain M. J., *42*, (1945): 18–25.

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A Study of a Site in Arizona Where a Dog Apparently Acquired a *Coccidioides Immitis* Infection

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ONLY A FEW PROJECTS have resulted in the isolation of *Coccidioides immitis* from soil. Such studies yield useful ecological data.

On Aug. 25, 1955, a family of two adults and three children, who had recently moved to Phoenix, Ariz., from the Midwest, took their 7-month-old Boxer pup with them on a picnic near Cactus, Ariz. The dog had been kept indoors almost constantly until then, but on this trip was active, running, digging, and inhaling for animal scents at many rodent burrows.

Eleven days later, the dog developed a fever and a cough. This was followed by anorexia, loss of weight, and diarrhea. When this condition did not respond to antibiotic therapy, coccidioidomycosis was suspected by the attending veterinarian. The coccidioidin test resulted in a 7-mm. indurated wheal and, when repeated three weeks later, the induration was 28 mm. The precipitin test was positive. Dissemination became evident early in November and the dog was euthanatized on Nov. 28, 1955. There were gross visible lesions in the lungs, liver, spleen, kidneys, and pericardium. Sporangia were demonstrated in lesion exudate, and *C. immitis* was recovered by culture methods.

When the diagnosis became definite, the owner and his family were advised that they also might have been exposed to the fungus, but they declined physical examinations and skin tests. None had developed any illness by December, when they moved from the vicinity.

The picnic site was visited and was found to have typical flora and fauna for the central Arizona desert (fig. 1). This paper reports on soil sampling, soil and air temperature determinations, as well as the rainfall for the area.

MATERIALS AND METHODS

Soil Sampling.—A total of 261 soil samples was collected, beginning Nov. 10, 1955. Specimens from ten similar sites made up each sample (fig. 2), which filled a pint-sized paper carton three quarters full. Surface specimens were collected by using the cartons as digging tools. Deeper sites were first exposed by a small shovel which was placed in isopropyl alcohol after each use.

The soil in each carton was well mixed and taken to the laboratory, where about 10 Gm. of the soil was suspended in 30 ml. of physiological saline solution (NaCl, 0.85%). This suspension was thoroughly mixed and was allowed to settle until the supernatant fluid was only slightly muddy. Using a pipette, 4 ml. of the supernatant fluid was drawn off and placed in a test tube. To this was added 1 ml. of saline solution containing 20,000 units of penicillin and 4,000 units of streptomycin. This was mixed and allowed to stand at least one hour. The contents of the tube were drawn into a 5-ml. syringe and 1.25-ml. aliquots were injected intraperitoneally into each of 4 mice.

All mice that died prior to 30 days were examined for sporangia of *C. immitis* if gross lesions were found. After 30 days, the rest of the mice were killed and examined for sporangia if gross lesions were found.

If sporangia were found, four tubes (15 x 125 mm.) of Sabouraud's dextrose agar were inoculated per mouse with a mixture of the lungs, liver, spleen and kidneys. Four tubes have been found more likely than a single tube to reveal a minimal infection. Confirmation consisted of gross and microscopic examination of the culture.

If no lesions were found, the lungs, liver, spleen, and kidneys were mashed together and were used for an inoculum for four tubes of Sabouraud's dextrose agar per mouse. These were incubated at 97 F. for six weeks. Any suspicious growth was examined grossly and microscopically and, if it appeared to be *C. immitis*, it was mixed with 4 ml. of saline solution and 1 ml. was injected intraperitoneally into each of 4 mice. Final confirmation consisted of finding sporangia in the tissues of these mice. They were held for 30 days, unless they died sooner.

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TABLE 1—Summary of Collection of Soil Samples from a Site Near Cactus, Arizona and the Yield of Coccidioides Immitis

Sampling dates	Types of soil samples (10 sites per sample)						Totals
	Not near rodent burrows			From rodent burrows			
	Surface	6 in. deep	12 in. deep	Surface	6 in. deep	12 in. deep	
Nov. 10, 1955	0/0	0/0	0/0	0/0	1/4	0/0	1/4
Nov. 27, 1955	0/7	0/5	0/2	5/9	4/7	0/2	9/31
Mar. 10, 1956	0/1	0/1	0/1	0/3	1/2	0/2	1/10
May 23, 1956	0/1	0/1	0/1	0/2	0/2	0/1	0/8
July 14, 1956	0/2	0/2	0/2	0/6	0/4	0/4	0/20
Aug. 22, 1956	0/1	0/1	0/0	0/2	0/2	0/2	0/8
Sept. 10,1956	0/2	0/2	0/2	2/4	2/5	0/5	4/20
Nov. 28, 1956	0/1	0/1	0/0	0/2	0/2	0/1	0/7
Mar. 15, 1957	0/1	0/1	0/1	0/3	0/2	0/2	0/10
May 20, 1957	0/1	0/1	0/1	0/3	0/2	0/2	0/10
July 5, 1957	0/2	0/2	0/2	0/10	1/10	1/9	2/35
Aug. 10, 1957	0/1	0/1	0/1	0/3	0/2	0/2	0/10
Sept. 3, 1957	0/2	0/2	0/2	4/10	2/10	3/10	9/36
Dec. 18, 1957	0/2	0/2	0/2	1/16	3/15	0/15	4/52
Totals	0/24	0/22	0/17	12/73	14/68	4/57	30/261

Numerator = No. of positive samples, denominator = No. of samples.

Soil and Air Temperature Determinations.—The air temperature was determined with a liquid-in-glass type thermometer in a louvered box.[11] The soil temperatures were taken by inserting the bulb of a liquid-in-glass type thermometer into the desired site, while insulating the remainder of the thermometer.

Precipitation Determinations.—The monthly figures were obtained from the U.S. Department of Commerce Weather Bureau station at the Phoenix airport, 14 miles southeast of the site under study.[12]

RESULTS

Soil Sampling.—From Nov. 10, 1955, through Dec. 18, 1957, 261 soil samples (representing soil from 2,610 different sites) were collected, and 30 (11.5%) yielded *C. immitis.* Samples of soil, 24 taken at the surface, 22 from 6 inches deep, and 17 from 12 inches deep, collected at least 3 ft. from rodent burrows, were all negative. However, 12 of 73 (16.4%) surface samples, 14 of 68 (20.6%) 6-inch-, and four of 57 (7.0%) 12-inch-deep samples taken directly from rodent burrows were positive. All the specimens found to be positive were collected in the September through December period, with the exceptions of one March and one July specimen in the 6-inch-deep samples and one July sample in the 12-inch-deep samples (table 1).

Soil and Air Temperature Determinations.—During 1957, on one day each month, a set of temperatures was taken at 5 a.m., 11 a.m., 5 p.m., and 11 p.m. A day

Fig. 1—The site where the study was conducted. The creosote bush Larrea tridentata is the predominant bush in the area.

was selected if the temperatures of the official forecast were to be close to the average for that month. If the actual maximum air temperature was within 2 degrees of the normal average maximum for the month, that set of temperatures was used; otherwise another set was collected. These temperatures are recorded (table 2).

Precipitation.—The monthly precipitation figures are recorded (table 3).

DISCUSSION

An over-all analysis of the climate and the relationship of the endemic area to the Lower Sonoran Life Zone has been presented elsewhere.[1,7-10] A number of unpublished soil-sampling studies by the Arizona State Department of Health indicate that this fungus can only be isolated from desert soil. Even in the desert, positive sites seem to be miles apart.

In this study, the findings of the positive samples in animal burrows correspond to the studies of others.[1-6] It is surmised that the burrow provides a better environment for the fungus than other sites in the desert soil. Perhaps the rodent manure provides enough extra nitrogen to help the fungus grow more abundantly.

The daytime summer temperatures, particularly of the soil closer to the surface, are revealing. They are believed to be too

TABLE 2—Temperatures at Site Near Cactus, Arizona, Involved in a Coccidioides Immitis Ecological Study

	1957 Temperatures (F.)												
Hour taken	Jan. 21	Feb. 5	Mar. 18	Apr. 20	May 23	June 7	July 9	Aug. 18	Sept. 20	Oct. 16	Nov. 6	Dec. 22	Average
AIR TEMPERATURES													
5 a.m.	33	39	46	54	68	72	79	78	77	72	51	42	59
11 a.m.	57	64	71	81	91	94	95	96	89	87	75	65	80
5 p.m.	54	55	62	68	85	100	102	101	98	84	65	56	78
11 p.m.	50	52	58	63	68	73	86	76	70	66	58	51	64
Average	49	53	59	67	78	85	90	88	84	77	62	51	70
SOIL TEMPERATURES ½ INCH BELOW SURFACE													
5 a.m.	27	34	50	56	72	73	79	76	76	64	49	38	58
11 a.m.	57	99	103	105	131	146	153	151	138	131	105	88	117
5 p.m.	48	51	68	77	98	138	154	145	136	99	62	49	94
11 p.m.	41	42	54	56	72	73	92	86	83	70	55	42	64
Average	43	57	69	74	93	108	120	115	108	91	68	54	83
SOIL TEMPERATUTES 2½ INCHES BELOW SURFACE													
5 a.m.	36	38	51	57	73	75	81	80	79	76	54	39	62
11 a.m.	49	50	70	83	109	115	120	118	111	100	65	50	87
5 p.m.	49	50	67	81	95	111	119	121	115	94	65	50	85
11 p.m.	45	44	58	58	81	81	96	93	91	82	58	43	69
Average	45	46	62	70	90	96	104	103	99	88	61	46	76
SOIL TEMPERATURES 6 INCHES BELOW SURFACE													
5 a.m.	44	46	55	59	81	81	86	85	84	80	60	47	67
11 a.m.	45	48	58	68	83	83	105	104	103	82	61	49	74
5 p.m.	49	49	67	77	88	90	110	109	108	94	67	49	80
11 p.m.	46	47	58	66	86	87	97	96	94	85	63	48	73
Average	46	48	60	68	85	85	100	99	97	85	63	48	74
SOIL TEMPERATURES 1 INCH DEEP IN RODENT BURROW													
5 a.m.	32	36	51	57	73	74	80	79	77	68	51	38	60
11 a.m.	53	78	84	91	116	123	136	133	121	113	78	73	93
5 p.m.	49	58	79	96	97	116	131	133	124	97	73	49	92
11 p.m.	43	49	57	65	78	79	93	92	85	83	71	42	70
Average	44	55	68	77	91	98	110	109	102	90	68	51	79
SOIL TEMPERATURES 6 INCHES DEEP IN RODENT BURROW													
5 a.m.	45	47	56	64	71	80	86	81	76	70	59	43	65
11 a.m.	51	53	68	73	83	86	98	96	93	79	66	49	75
5 p.m.	52	54	65	69	84	93	98	96	95	84	67	53	76
11 p.m.	49	49	57	65	73	81	88	82	76	71	60	49	67
Average	49	51	62	68	78	85	93	89	85	76	63	49	71
SOIL TEMPERATURES 12 INCHES DEEP IN RODENT BURROW													
5 a.m.	45	48	56	65	72	82	88	87	79	71	61	45	67
11 a.m.	49	51	66	70	81	84	92	90	89	81	69	48	73
5 p.m.	50	53	67	72	83	86	94	91	89	84	70	48	74
11 p.m.	48	48	57	68	79	82	90	84	82	76	61	47	69
Average	48	50	62	69	79	84	91	88	85	78	65	47	71

TABLE 3—Precipitation in Inches by Months for the Years 1954–1957

Years	Months												Totals	Average per month
	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.		
1954	0.88	0.58	1.22	0.02	0.11	0.02	0.36	0.55	0.44	0.12	T*	0.01	4.31	0.36
1955	2.41	0.09	T	T	0.02	0.95	4.19	1.80	T	0.13	0.05	0.18	9.82	0.82
1956	0.67	0.64	0.00	0.03	T	0.01	0.92	0.46	0.02	0.06	0.00	0.01	2.82	0.24
1957	1.57	0.21	0.53	0.12	0.43	0.26	0.72	0.85	0.00	2.66	0.02	0.23	7.60	0.63
Average (1954–1956)	1.38	0.38	0.44	0.04	0.14	0.31	1.55	0.92	0.12	0.74	0.02	0.11	6.14	0.51
Mean (1900–1955)	0.82	0.78	0.66	0.40	0.13	0.08	1.02	1.07	0.78	0.41	0.65	0.85	7.65	0.64

Figures are taken from records of the U.S. Department of Commerce Weather Bureau Station at the Phoenix Municipal Airport, which is 14 miles southeast of the site involved in the *Coccidioides immitis* study. The altitude and terrain are similar.

*T = trace.

high for the fungus to live for many days. The two positive specimens were secured a few days after a shower, which could possibly have started fungal growth from dormant spores or mycelia still living in the deeper soil.

However, the summer temperatures in the rodent burrow at the 6-inch depth are in a range that is believed to be favorable to the fungus. It is not surprising that 14 of the 30 positive samples were from this type of location.

The summer to early fall daytime temperatures at the surface of the rodent burrow (table 2) seem too high for the fungus to survive long.

At no time was an air temperature and only once was a soil temperature recorded that was below freezing.

This area has two rainfall seasons, one from November to March, the other in July and August (table 3).

CONCLUSIONS

It is surmised that the period of April into July is a period in which the high soil temperatures and low moisture content destroy much of the surface microflora of the desert. When the rains come in July and August, the few spores or mycelia of *C. immitis* that are still viable start new growth. This results in soil which is more infective in the early autumn than at any other time of the year.

Dust storms are common in the fall in this area, and more cases of coccidioidomycosis are reported in man and animals at this time of the year.

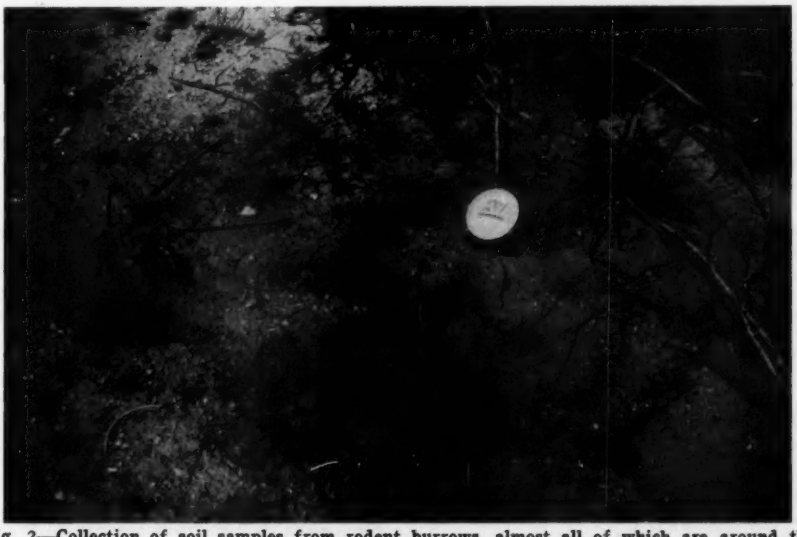

Fig. 2—Collection of soil samples from rodent burrows, almost all of which are around the bases of creosote bushes.

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When buildings are constructed in the endemic area, the soil in certain areas can provide a heavy infection potential when it is graded, shoveled, pushed, and blown about, particularly in the fall of the year.

SUMMARY

A site in the Arizona desert where a dog apparently acquired coccidioidomycosis was studied for two years. Soil samples 24 from the surface, 22 from the 6-inch depth, and 17 from the 12-inch depth, all from areas not near rodent burrows, collected at intervals over a two-year period were all negative for *Coccidioides immitis*.

Of soil samples collected directly from rodent burrows, 12 of 73 from the surface, 14 of 68 from the 6-inch depth, and four of 57 from the 12-inch depth yielded *C. immitis*. Almost all positive samples had been collected in the September through December period. Surface soil temperatures were too high in the early summer to be favorable for the growth of many microorganisms.

The greater number of human and animal cases of coccidioidomycosis was reported in this area in the fall, following the late summer rains.

References

[1] Ajello, L., Reed, R. E., Maddy, K. T., Budurin, A. A., and Moore, J. C.: Ecological and Epizootiological Studies on Canine Coccidioidomycosis. J.A.V.M.A., *129*, (1956): 485–490.

[2] Davis, B. L., Smith, R. T., and Smith, C. E.: An Epidemic of Coccidioidal Infection (Coccidioidomycosis). J. Am. M. A., *118*, (1942): 1182–1186.

[3] Egeberg, R. O., and Ely, A. F.: *Coccidioides Immitis* in the Soil of the Southern San Joaquin Valley. In "Therapy of Fungus Diseases," T. H. Sternberg and V. D. Newcomer, ed. Little, Brown & Co., Boston (1955): 311–315.

[4] Egeberg, R. O., and Ely, A. F.: *Coccidioides Immitis* in the Soil of the Southern San Joaquin Valley. Am. J. M. Sci., *231*, (1956): 151–154.

[5] Elconin, A. F., Egeberg, R. O., and Lubarsky, R.: Growth Patterns of *Coccidioides Immitis* in the Soil of an Endemic Area. Proc. Symp. on Coccidioidomycosis, Phoenix, Ariz., Feb. 11–13, 1957. U.S. Dept. of Health, Education and Welfare, Public Health Service, Communicable Disease Center, Atlanta, Ga. Pub. Health Serv. Publ. 575 (1957): 168–170.

[6] Emmons, C. W.: Isolation of *Coccidioides Immitis* from Soil and Rodents. Pub. Health Rep., *57*, (1942): 109–111.

[7] Maddy, K. T.: Coccidioidomycosis Symposium. Arizona Public Health News, *50*, 1957.

[8] Maddy, K. T.: Ecological Factors of the Geographic Distribution of *Coccidioides Immitis*. J.A.V.M.A., *130*, (June 1, 1957): 475–476.

[9] Maddy, K. T.: Ecological Factors Possibly Relating to the Geographic Distribution of *Coccidioides Immitis*. Proc. Symp. Coccidioidomycosis, Phoenix, Ariz., Feb. 11–13, 1957. U.S. Dept. of Health, Education and Welfare, Public Health Service, Communicable Disease Center, Atlanta, Ga. Pub. Health Serv. Publ. 575, (1957): 144–157.

[10] Maddy, K. T.: The Geographic Distribution of *Coccidioides Immitis* and Possible Ecologic Implications. Arizona Med., *15*, (1958): 178–188.

[11] U.S. Department of Commerce, Weather Bureau: Instructions for Climatological Observers. Circular B, 10th ed. U.S. Govt. Print. Off. (Oct., 1955): 7–18.

[12] U.S. Department of Commerce, Weather Bureau: Local Climatological Data with Comparative Data, 1953–1957, Phoenix, Ariz., Sky Harbor Airport. NWRC Asheville, N. Car., 1954–1958.

Cataracts in Chickens with Lymphomatosis

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CONSIDERABLE INTEREST in the eye has occurred since Gregg,[8] in 1941, demonstrated a high incidence of malformations in infants whose mothers had rubella during the early months of pregnancy. Cataracts were one of the abnormalities considered to result from this virus infection. Cataracts may follow distemper in pups, especially when they are affected before or immediately after weaning.[11]

Blattner *et al.*[1, 12] have observed defective development of the lens in chicken embryos infected with Newcastle disease and the influenza A viruses. Ocular lesions have occurred in chickens infected with the virus of lymphomatosis.[5, 9] In these chickens there is an iritis and iridocyclitis characterized by an infiltration of lymphocytes. The iris frequently shows a depigmented circle around the pupil, synechiae are present, and the pupil often becomes constricted. In extreme cases, the pupil may disappear completely. One or both eyes may be involved. Nelson and Thorp[10] reported that "no involvement of the lens itself has been observed microscopically" in ocular lymphomatosis. Findlay and Wright[6] did not report any involvement of the lens in their study of ocular lesions in epizootic blindness of fowl.

In 1944, DeVolt[4] observed cataracts in 9 chickens in a flock of 50. In most cases, the opacities were bilateral and varied in degree from a slight clouding to a distinct grayish-white patch located near the center of the lens. Lenses that were removed and fixed showed a tendency to liquefaction in the cataract zone. DeVolt[4] suggested that two causative factors could be considered: first, a strong light; second, a ration altered by wartime conditions.

In 1958, Flowers *et al.*[2, 7] reported the occurrence of cataracts and iridocyclitis in association with encephalomyelitis in several flocks of chickens. The incidence of cataracts was 35 per cent in some flocks. The blindness usually was bilateral. Occasionally a bird also showed either bilateral leg weakness or a tremor of the head and neck. Attempts to isolate a virus were unsuccessful. Pathological changes, characterized by lymphocytic infiltration, were present in the iris, ciliary body, and about the canal of Schlemm. Lymphocytic infiltration also occurred in the brain and spinal cord. Necrosis was present in the lens, and lymphoid hyperplasia was evident in the viscera of some chickens.

The virus of lymphomatosis is known to be transmitted to the embryo through the egg, and also directly from chick to chick.[3] Recently, an opportunity occurred in our laboratory for study of cataracts in chickens apparently infected with the virus of lymphomatosis. The experimental observations are reported here.

MATERIAL AND METHODS

The hen (11960) with the original cataract was a normal-appearing, single-combed White Leghorn, approximately 1 year old when received from the Poultry Department of Texas A. & M. College with 15 other hens and 2 roosters of similar breed. A cataract in the left eye was first observed several weeks after she was received. She was mated when approximately 18 months old and kept under observation until killed 18 months later.

The White Leghorn male (1266), 7 months old when mated with this female, was 1 of the chickens hatched from the original group of 16 hens. His eyes were grossly normal at the time of the mating; the pupils were first observed to be slightly irregular when he was 498 days old. He was observed until killed 258 days later.

The eggs were collected each day and put into the incubator. The 29 chicks which hatched were kept in small batteries and pens with others of similar age. The older birds were isolated in individual cages and, when mated, were put into a small pen. They were fed a commercial ration. The eyes were examined routinely with a dissecting microscope and some with an ophthalmoscope. When the chickens died or were killed, the eyes were removed and usually photographed. They were cut transversely, and the inner surfaces were examined with the dissecting microscope. Some were examined with a polarizing microscope. Paraffin sections were prepared for histological study. Viscera from some of the chickens were studied. Sections were stained routinely with hemotoxylin and eosin. Selected sections were stained by the Mallory aniline blue and the periodic acid-Schiff techniques.

RESULTS

The genealogy of first-generation chickens that developed eye abnormalities is shown (chart 1). Of the 29 chickens from the first mating, 8 developed an iritis; 6 of these also had irregular pupils and 5 had

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cataracts. An irregular pupil was first observed after experiment day 121, and the last after 196 days. On experiment day 51, the right lens of 1 chicken had a localized area of pigmentation which persisted throughout life. Four of the chickens without eye abnormalities were kept under observation for eight to 12 months.

The cataract in the left eye of the original female (11960) involved approximately two thirds of the lens when she was 1 year old; a year later, it involved the entire lens (fig. 1). Many small crystals were present in the cataract. As the bird aged, cracks developed on the anterior surface of the cataract. Some excess fluid accumulated in the anterior chamber during the later months. No indication of an inflammatory process was observed in either eye. The hen was killed when approximately 3 years old and, except for the cataract, seemed normal.

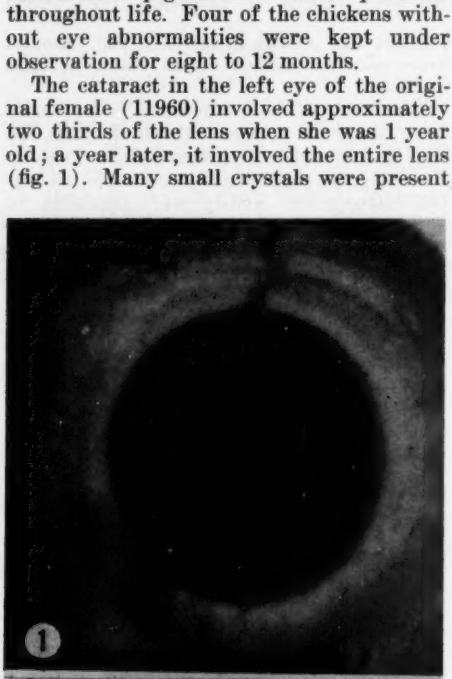

Fig. 1—Eye of hen 11960, approximately 3 years old, showing a cataract involving the greater part of the lens; at the age of 1 year, it involved two thirds of the lens.

Fig. 2—Crystals from the cataract shown in figure 1. The lens was dabbed onto a slide and, after drying, it was examined with polarized light. These crystals were blue and, when rotated 90 degrees, their color changed to yellow. Polarized light; x 420.

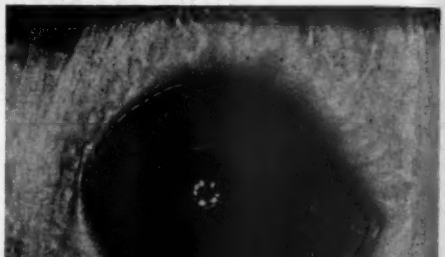

Fig. 3—Eye of chicken C-35 with iritis showing a typical irregular pupil.

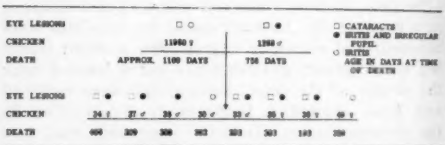

Chart 1—Cataracts and iritis in parental and first-generation chickens. Of 29 chickens hatched from the first mating: 8 were normal and were discarded when 142 to 244 days old; 6 died from causes unknown when 71, 75, 82, 104, 188, and 189 days of age; 3 died from suffocation when 86, 123, and 131 days old; and 4 killed when 260, 401, 578, and 583 days old had developed no eye lesions.

The posterior chamber of the left eye was filled with a firm grayish-white mass that "shelled" out easily. The anterior surface of the mass was flat and the distorted lens and portions of the iris were attached to it. Several irregular cystlike spaces, filled with brownish fluid, were present within the ossified mass. Crystals were present, some of which were either yellow or blue in polarized light and, when rotated 90 degrees, the color changed from

either a blue to yellow or vice versa (fig. 2). Lymphocytes were present in the iris and ciliary body of both eyes. The lens from the left eye was not studied histologically; the right was normal. A hemangioma-like tumor, 6 cm. in diameter, was present in the area of the ovary.

The eyes of male 1266 were considered normal until the 498th day, when the pupils were observed to be irregular. Subsequently, the right anterior chamber became cloudy. This male was killed when 756 days old. The anterior chamber of the right eye was cloudy and the pupil was irregular (fig. 3). Fibrous adhesions were present between the iris and lens on the left. Many lymphocytes and a few eosinophils were present in the iris and in the anterior chamber of each eye (fig. 4). The lens in one eye was normal and the other had focal areas of cystic degeneration (fig. 5).

The left pupil in chicken C-24 was irregular on experiment day 196, and a month later the right pupil was irregular. Adhesions developed between the iris and lens. This hen appeared weak on the 406th day and was killed. Lymphocytes were present in the iris, ciliary body, and choroid. Degenerative changes characterized by vacuolization and fragmentation of the lens fibers were present in the right lens. The left lens was normal.

On experiment day 184, the pupil in the right eye of chicken C-27 appeared fixed

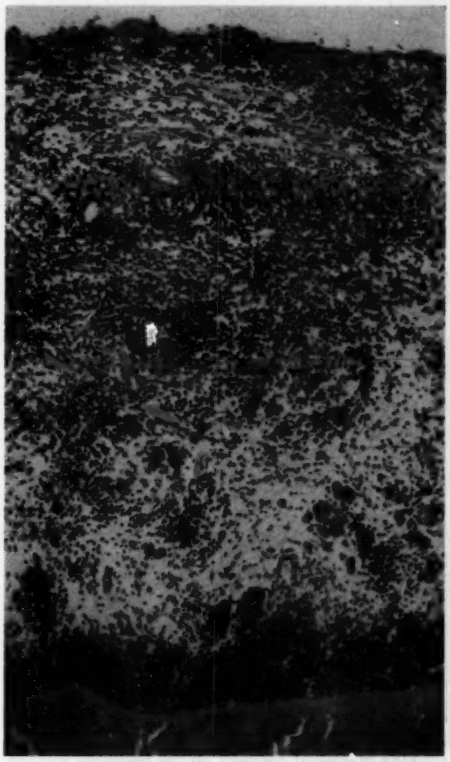

Fig. 4—Section of iris of chicken C-1266 showing numerous lymphocytes and eosinophils. H & E stain; x 320.

Fig. 5—Focal area of degeneration in the lens of chicken C-1266. This is a morgagni-type cataract. Arrows indicate artifacts resulting from preparation of section. H & E stain; x 110.

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and the anterior chamber was cloudy; three weeks later, the pupil was ellipitical. Focal areas of opacity were present in the left lens at this time. This chicken died on the 215th day. Masses of black pigment were present on the anterior and posterior surfaces of both lenses. Only one eye was studied histologically. There were adhesions between the iris and the lens. Lymphocytes were present in the iris and the

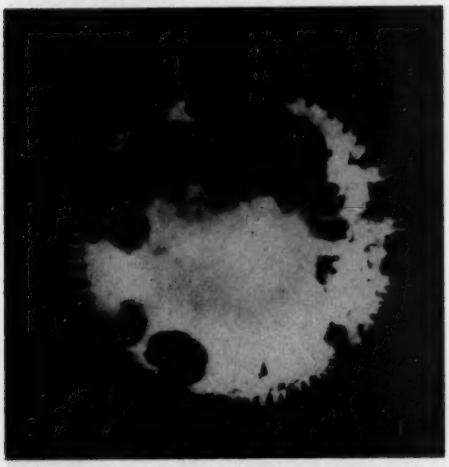

Fig. 6—Posterior view of the lens of chicken C-33 showing masses of pigment on the posterior surface.

lens was degenerated. The epithelial cells in the area of the capsule on the anterior surface had proliferated. Irregular spaces filled with hyaline-like material and vacuoles were present in the lens.

The anterior chambers of the right eye of chicken C-28 were cloudy on experiment day 134. A month later, the pupil in each eye was irregular. When killed on the 308th day, masses of pigment were present on the posterior surface of each lens. Adhesions were present between the lens and iris. Lymphocytes infiltrated the iris, heart, kidneys, liver, lungs, and testicles. The lenses were normal.

The eyes of chicken C-30 were normal except for a small area of pigment either in or on the right lens which was first observed on day 51. This chicken was killed on day 382. The area in the right eye resembled a small ball of cotton with granules of reddish-brown pigment attached to it. A few lymphocytes were present in the iris and choroid. No changes were observed in the lens.

Opacities were present in the lenses of chicken C-33 on the 125th day. These progressively increased in size and the bird appeared completely blind on the 292nd day, at which time he was killed. The pupils were irregular and the right anterior chamber was so cloudy that the pupil could not be seen. Small crystals were present in the lenses, and pigment partially covered both the anterior and posterior surfaces (fig. 6). Both lenses were degenerated. The center of the left lens was filled with a pink-staining granular material and the anterior-posterior diameter was markedly decreased (fig. 7). The epithelial cells in the capsule had proliferated (fig. 8) and fibroblasts extended from the capsule down into the degenerated portion of the lens (fig. 9). Lens fibers were absent in focal areas in the right lens. Mononuclear cells were present in focal areas (fig. 11). Eosinophilic-staining granules that varied in size were present in the cytoplasm of these mononuclear

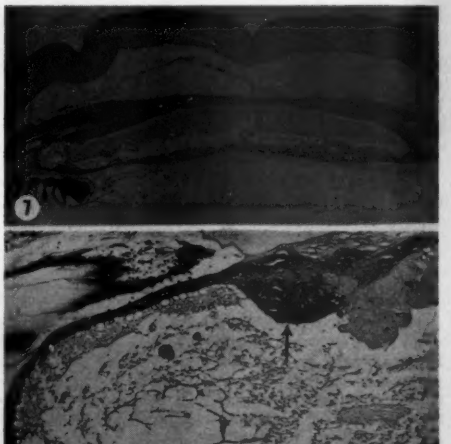

Fig. 7—Sagittal section of lens of chicken C-33 showing extensive degeneration and a decrease in the anterior-posterior diameter. The iris is infiltrated with lymphocytes. H & E stain; x 45.

Fig. 8—Sagittal section of a portion of lens of chicken C-33 which is completely liquid. Groups of degenerated epithelial cells project inward from the capsule. H & E stain; x 85.

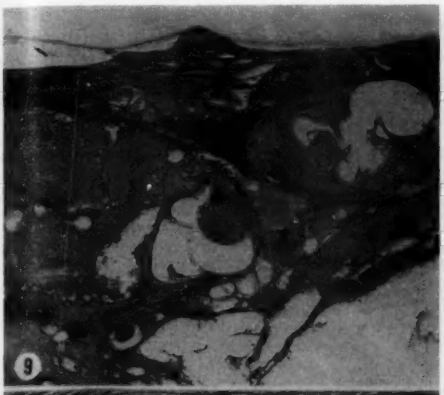

Fig. 9—Fibroblasts in the capsule of the lens proliferate and extend into the degenerated lens of chicken C-33. H & E stain; x 215.

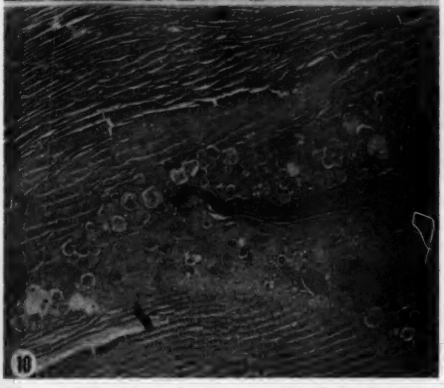

Fig. 10—Focal area of degeneration in the lens of chicken C-33 with many epithelial cells. H & E stain; x 75.

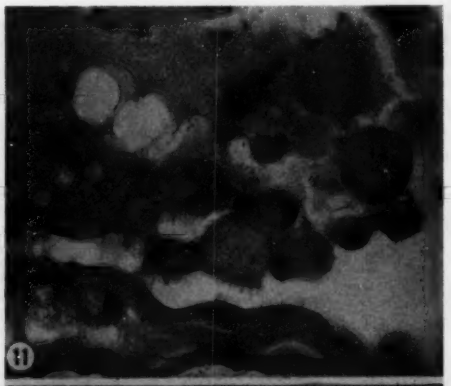

Fig. 11—A higher magnification of the cells shown in figure 10. H & E stain; x 535.

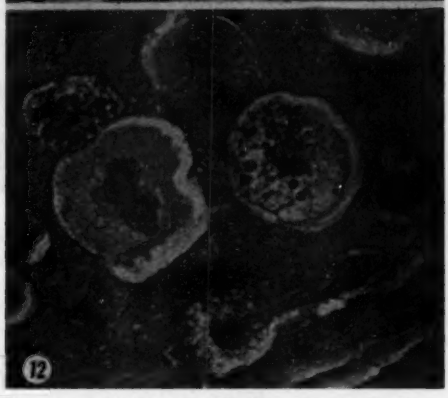

Fig. 12—Eosinophilic granules are present in the cytoplasm of some of the epithelial cells in the degenerated lens of chicken C-33. They resemble fragments of lens protein. H & E stain; x 535.

cells (fig. 12). Many lymphocytes infiltrated the iris and ciliary body of both eyes and adhesions were present between the lens and iris.

The left pupil in chicken C-35 was irregular on day 165, and a month later the right was similarly affected. Adhesions were present. The anterior chamber of the right eye was cloudy when the bird was killed on day 303. Focal areas of pigmentation occurred on the lenses similar to those previously described. Many lymphocytes were present in the iris, ciliary body, and choroid. Similar cells were present in the liver, kidneys, and intestines. The left lens had focal areas of cystic degeneration; the right lens was normal.

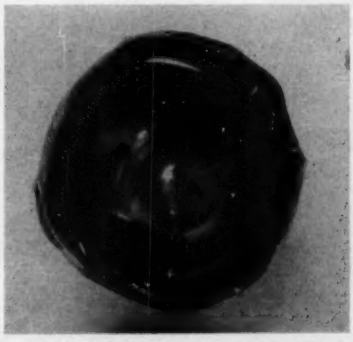

Fig. 13—The detached retina covers the greater part of the posterior surface of the lens (A) of chicken C-36.

Fig. 14—Chicken C-36 showing extensive degeneration of the lens (A), the iris infiltrated with lymphocytes (B), cornea (C), chronic inflammation in the area of the suspensory ligament (D), and a portion of the detached retina (E). H & E stain; x 35.

Both anterior chambers of chicken C-36 were cloudy and the pupils were small and irregular on day 121. Adhesions subsequently developed between the iris and the lens. This bird was killed on day 198. The greater part of the retina in the left eye was detached (fig. 13). Focal areas of fibrosis and hyperplastic epithelial cells were present along the inner surface of the choroid. Fibrous adhesions occurred in the area of the suspensory ligament (fig. 14). The capsule of the lens was fibrotic along the posterior surface. Some of this fibrous tissue extended from the capsule into the

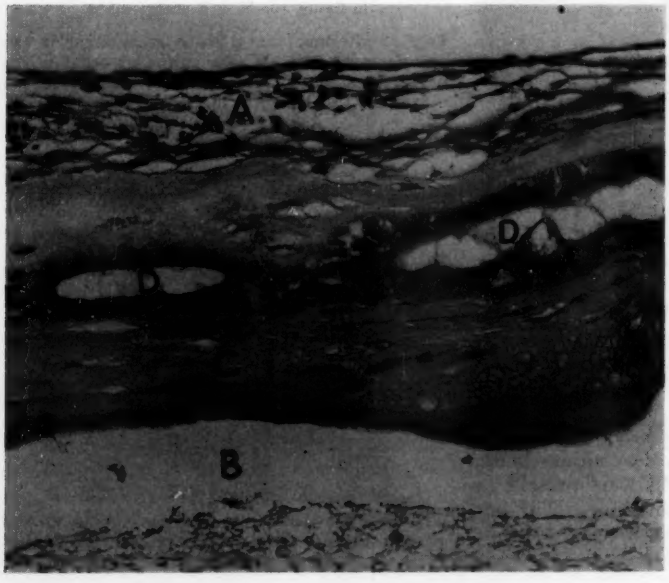

Fig. 15—Eye of chicken C-33 showing (A) iris with few lymphocytes; (B) the completely liquid lens; (C) the capsule of the lens adhering to the iris; and (D) cyst-like spaces in the capsule resulting from a proliferation of the epithelial cells. H & E stain; x 320.

lens. In focal areas, the epithelial cells of the capsule were proliferated, appearing as a duplication of the lens capsule (fig. 15). The degeneration within the lens was similar to that previously described. The granules in the cytoplasm of the cells stained dark red with the periodic acid-Schiff technique. Lymphocytes and eosinophilic leukocytes were present in the iris, ciliary body, and choroid. A few lymphocytes infiltrated the optic nerve and harderian gland. No changes were observed in the cornea.

Chicken C-49 was a normal female that died on the 329th day, following the local application of *d*-tubocurarine chloride (Intocostrin*) to the eye. Lymphocytes were present in the iris and ciliary body. An ovarian tumor, 5 cm. in diameter, composed of lymphocyte-like cells was present.

Discussion

The clinical and pathological findings in this group of chickens are typical of lymphomatosis. In addition to the ocular manifestations, some of the birds showed lymphocytic tumors and an infiltration of the viscera with similar cells. Meningoencephalitis and ataxia occurred in some.

The degree of degeneration in the lens varied; usually it was bilateral. The less extensive lesions were characterized by irregular vacuolated areas that did not take the eosin stain. The more extensive lesions were characterized by liquefaction of the entire lens, except for a narrow pink-staining zone adjacent to the capsule. Epithelial cells in the capsule proliferated in focal areas, and fibrous tissue grew from the capsule down into the degenerating lens.

A third or intermediate type of lens degeneration was characterized by focal cystic areas and vacuolization of the lens fibers. In such areas, there were mononuclear cells with pink-staining granules of various sizes within their cytoplasm. These cells may represent degenerating lens epithelium. Since there is no vascular system within the lens, one would not expect to find macrophages originating from the circulation to be within a degenerating lens. The eosinophilic granules in the cytoplasm may represent only degenerating lens protein and not phagocytized material.

* Intocostrin is produced by E. R. Squibb and Sons, Division of Mathieson Chemical Corp., New York, N.Y.

A lymphocytic infiltration occurred in all ocular structures, including the periorbital fat, muscle, and lacrimal gland. Iritis or degeneration of the lens, or both, were present in one or both eyes of several chickens. The eye lesions were the same, except for the degeneration of the lens, as

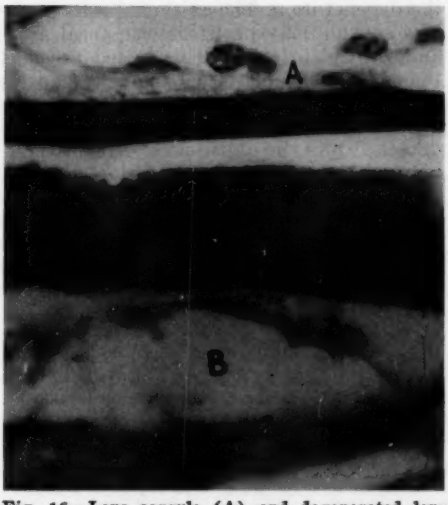

Fig. 16—Lens capsule (A) and degenerated lens (B) of chicken C-36. Some of the epithelial cells in the area of the capsule have PAS-positive granules in their cytoplasm. Periodic acid-Schiff; x 1296.

Nelson and Thorp [10] described in 1943 as being typical for ocular lymphomatosis. The lens degeneration was the same as that described recently by Bridges and Flowers [2] in chickens with encephalomyelitis. These observations support the opinion that a virus was the causative agent for these cataracts, as well as for avian lymphomatosis.

This study indicates that the virus of lymphomatosis may enter the lens from the fluid within the eye and result in coagulation necrosis of the fibers. No lymphocytic reaction was present in the lens. Blindness may result from these cataracts. However, birds with iritis may survive for an indefinite period and appear normal otherwise. Retinal detachment and ossification may result from this ocular infection.

Summary

Ocular lesions are known to occur in chickens infected with the virus of lymphomatosis; however, degeneration of the

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lens with typical cataracts apparently has not been considered as a characteristic manifestation of this infection.

The lesion in the lens is characterized by either a focal or diffuse degeneration. The lens fibers apparently undergo coagulation necrosis, followed by cyst formation. There is no inflammatory reaction associated with the degeneration. The epithelial cells in the lens capsule may proliferate in focal areas and project into the degenerated areas of the lens. Fibroblasts in the lens capsule may also proliferate and extend into the degenerated lens. The retina may become detached and, in old lesions, osteoid tissue may develop within the posterior chamber.

The virus may enter the lens from contaminated fluid present in the anterior chamber. Chickens showing this lens lesions usually have an associated iritis. Some of the birds also show the lesion of visotrophic and neurotrophic lymphomatosis.

References

[1] Blattner, Russell J., and Williamson, Alice Polk: Developmental Abnormalities in the Chick Embryo Following Infection with Newcastle Disease Virus. Proc. Soc. Exptl. Biol. & Med., *77*, (1951): 619–621.

[2] Bridges, Charles H., and Flowers, A. I.: Iridocyclitis and Cataracts Associated with an Encephalomyelitis in Chickens. J.A.V.M.A., *132*, (Jan. 15, 1958): 79–84.

[3] Burmester, B. R., Gentry, R. F., and Waters, N. F.: The Presence of the Virus of Visceral Lymphomatosis in Embryonated Eggs of Normal Appearing Hens. Poult. Sci., *34*, (1955): 609–617.

[4] DeVolt, H. M.: Lamellar Cataracts in Chickens. Poult. Sci., *23*, (1944): 346–348.

[5] Doyle, L. P.: Neuritis or Paralysis in Chickens. J.A.V.M.A., *72*, (Feb., 1928): 585–587.

[6] Findlay, G. M., and Wright, Joyce: Ocular Lesions in Epidemic Blindness of Fowls. J. Comp. Path. & Therap., *46*, (1933): 139–148.

[7] Flowers, A. I., Grumbles, L. C., DuBose, R. T., and Delaplane, J. P.: Cataracts: A New Flock Problem in Chickens. Poultry Sci., *37*, (1958): 420–422.

[8] Gregg, N. McAlister: Congenital Cataract Following German Measles in the Mother. Tr. Ophth. Soc. Australia, *3*, (1941): 35–46.

[9] Lee, C. D., and Wilcke, H. L.: Transmission Experiments with Iritis of Fowls. Am. J. Vet. Res., *2*, (July, 1941): 292–294.

[10] Nelson, Norman M., and Thorp, Frank: Ocular Lymphomatosis with Special Reference to Chromatism of Irides. Am. J. Vet. Res., *4*, (July, 1943): 294–304.

[11] Smythe, R. H.: Veterinary Ophthalmology. Bailliere, Tindall and Cox, London (1956): 283.

[12] Williamson, Alice Polk, Simonsen, Lydia, and Blattner, Russell J.: Specific Organ Defects in Early Chick Embryos Following Inoculation with Influenza A Virus. Proc. Soc. Exptl. Biol. & Med., *92*, (1956): 334–337.

The Effect of Para-Aminobenzoic Acid on the Toxicity of 3-Nitro-4-Hydroxyphenylarsonic Acid in Chicks and Poults

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ALTHOUGH THE USE of organic arsenicals has been well established in medical practice for several hundred years, they became factors in the poultry and livestock industry following the report by Morehouse and Mayfield [5] that the inclusion of certain organic arsenicals either in the drinking water or the feed of chicks had both a coccidiostatic and growth-promoting effect. Since that time, numerous workers have confirmed these findings and extended their observations to other species. Frost and Spruth [2] have prepared an excellent review on the feed use of arsenicals.

Currently, 3-nitro-4-hydroxyphenylarsonic acid (3-nitro), arsanilic acid, and sodium arsanilate are added to feeds for growth stimulation; 3-nitro and the trivalent arsenosobenzene are used for coccidiosis control and 4-nitro-phenylarsonic acid is used for the prevention and cure of blackhead in turkeys.

Concomitant with the use of 3-nitro in feeds, there have been reports of transitory toxicosis in poultry and animals given these feeds. Milligan *et al.*[4] reported that, under commercial conditions, broilers fed 45.5 Gm. of 3-nitro per ton of feed occasionally displayed "an unsteadiness in walking on the part of a few individuals." Feed manufacturers frequently receive complaints that birds eating feed containing 3-nitro display a slight ataxia, incoordination, and a reluctance to walk. These signs are accentuated when the birds are disturbed. "Tired legs" is a term frequently used to describe this condition.

Sandground and Hamilton[10] reported the results of a series of tests showing the ability of para-aminobenzoic acid (PABA) to counteract the toxicity of several pentavalent organic arsenicals to rats. Furthermore, it was demonstrated [6, 9, 12] that the detoxification of these arsenicals did not counteract their trypanocidal effect.

The toxicity of organic arsenicals limits their use both in feeds and as medicaments. A method of detoxifying these arsenicals that did not interefere with their growth-promoting and medicinal value would be of considerable benefit. Therefore, studies were undertaken to determine if a single oral dose of PABA would protect chicks and poults from an ordinarily toxic dose of 3-nitro.

MATERIALS AND METHODS

Vantress-cross chicks, 4 weeks of age or older, with an average weight near 400 Gm., were used in the chick studies. In the turkey trials, Broad-Breasted Bronze poults, 4 weeks of age or older, with an average weight near 1,000 Gm., were used. Only birds appearing to be in good health were used in the experiments. The diets fed prior to the initiation of and during each experiment met or exceeded the nutritive requirements established by the National Research Council.[1]

All solutions were made up in 1 per cent sodium bicarbonate to provide 10 mg. of 3-nitro or PABA per milliliter. In some instances, a few drops of concentrated sodium hydroxide was added to obtain complete solution. Wing-banded birds, individually weighed, were given the predetermined dose of PABA or 3-nitro, or both, on the basis of weight. All solutions were injected into the crop with a syringe and a short piece of plastic tubing.

The arsenical was administered 30 minutes after PABA. This time period was based on the observation that administration of PABA within a three-hour period prior to giving $l.d._{50}$ of arsanilic acid counteracted the toxic effect.[7]

The 3-nitro was given to chicks at levels of 100 and 200 mg. per kilogram of body weight and to poults at levels ranging from 20 to 80 mg. per kilogram. The PABA was given at the same level as the subsequent dose of 3-nitro, based on the report of Sandground and Hamilton.[10]

All birds were maintained in metal batteries equipped with raised wire floors. Feed and water were provided ad libitum. Ten birds were used for each treatment. Straight-run chicks and poults were used in all experiments except experiment 1, in which male chicks were used.

Survival time of all birds in each experiment was determined. The following scoring method was used: Birds dying on the day of treatment or found dead at 8 o'clock the next morning were scored 1. Birds dying before 8 a.m. of the second day following treatment were scored 2, birds dying

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Presented in part at the 1957 meeting of the American Institute of Nutrition (Abstr. in Fed. Proc., 16, 1957: 1725).

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before 8 a.m. of the third day were scored 3, and birds dying after 8 a.m. of the third day were scored 4. Thus, the larger the average value for a group, the longer the survival time.

Each bird dying during the three-day observation period was examined for evidence of damage to the esophagus and crop and for the presence of fluid in the trachea. In no instance was trauma found, nor was there evidence of pneumonia.

EXPERIMENTAL PROCEDURE AND RESULTS

CHICK STUDIES

The results of all chick experiments are tabulated (table 1) and summarized (table 2).

Dosage with the sodium bicarbonate solution in the same manner, and in amounts equal to that which would have been used had PABA been given, had no effect on survival time. At the 100-mg. level of 3-nitro, some mortality occurred both in the presence and absence of a prior dose of PABA. Weight losses were greater in birds which received PABA.

Experiment 3.—In this experiment, the 200-mg. level of 3-nitro was lethal, regardless of prior treatment. At the 100-mg. level, PABA was of no benefit from the standpoint of weight loss or livability. Prior administration of the sodium bicarbonate solution did not alter the toxicity of 3-nitro.

TABLE 1—Effect of Para-Aminobenzoic Acid and $NaHCO_3$ Solution on Growth and Livability of Chicks Given 3-Nitro-4-Hydroxyphenylarsonic Acid

PABA (mg./kg.)	3-Nitro (mg./kg.)							
	0		100			200		
	Exper. 1	Exper. 3	Exper. 1	Exper. 2	Exper. 3	Exper. 1	Exper. 2	Exper. 3
0	.....	+74.0*(100)	−39.3(80)	−11.0(40)	−64.8(70)	.....(0)	.....(0)	.....(0)
100	+53.0(100)	.....	−27.0(100)	−33.0(30)	−69.3(40)	.....	.....	.....
200	+45.7(100)	.....	.....	.....	.....	−55.6(40)	.....(0)	.....(0)
400	.....	.....	.....	.....	.....	.....	−59.0(30)	.....
$NaHCO_3$								
100	.....	.....	.....	−78.1(70)	.....	.....	.....	.....
200	.....	.....	.....	.....	.....	.....	.....	−130.0(10)
400	.....	.....	.....	.....	.....	.....	.....(0)	.....

* Average change in body weight (Gm.). Percentage of survival given in parentheses.

Experiment 1.—There was no mortality among the birds given 100 mg. of 3-nitro per kilogram of body weight when this was preceded by PABA. Of the 10 birds given 200 mg. per kilogram preceded by PABA, 6 died. When PABA was not included in the treatment, 100 per cent mortality occurred at the 200-mg. level of 3-nitro and 20 per cent mortality at the 100-mg. level. Alone, PABA had no effect.

Experiment 2.—A second experiment was conducted to extend these observations and to determine if the vehicle in which the PABA was included may have influenced the results in the first test. Contrary to the results obtained in the first test, PABA did not prevent 100 per cent mortality when given with 3-nitro at the 200-mg. level. Dosage with PABA at a level of 400 mg. per kilogram of body weight followed by 200 mg. of 3-nitro appeared to offer some (30%) protection. The loss of body weight of survivors during the three-day observation period was quite similar to that of chicks given the 200-mg. level of 3-nitro and PABA in the first test.

When the combined mortality data from all tests were examined by the *chi*-square method,[11] the effect of PABA was not significant at the 100-mg. level of 3-nitro. A highly significant effect from PABA was obtained at the 200-mg. level ($X^2 = 6.43 > 1\% < 2\%$). Administration of PABA increased length of survival time, especially at the 100-mg. level of 3-nitro (table 2).

The sex of all birds was determined in the third experiment. Although distribution of males and females in each lot varied, 58.6 per cent of the males and 49.8 per cent of the females survived. This suggests a sex influence in tolerance to toxic levels of 3-nitro, and may offer an explanation for the unduplicated livability obtained in the first experiment, in which only males were used.

Approximately 30 minutes after administration of 3-nitro, a marked decrease in physical activity of the chicks was noticed. Most of the chicks were completely inactive two hours after treatment, moving from a drooped, squatting position only when forced. Chicks which succumbed continued

to be inactive, with heads and necks retracted and feathers ruffled, becoming comatose and dying eight to 72 hours after treatment. Survivors usually regained some activity eight to 24 hours after administration of 3-nitro and appeared to be normal after 72 hours. Some birds which, on the basis of activity, appeared to be recovering, died suddenly without recurrence of signs observed in the first few hours following treatment.

TABLE 2—Summary of Chick Experiments

Treatment	No. treated/ No. survived	Survival (%)	Av. survival time of birds dying (days)	Av. change in weight (Gm.)
100 mg. 3-nitro + PABA	30/17	56.6	2.81	−43.1
200 mg. 3-nitro + PABA	40/7	17.5	1.64	−57.0
100 mg. 3-nitro	40/26	65.0	1.99	−48.3
200 mg. 3-nitro	50/1	02.0	1.53	−130.0
100 mg. PABA	10/10	100.0	.....	+53.0
200 mg. PABA	10/10	100.0	.....	+45.7
none	10/10	100.0	.....	+74.0

Experiment 4.—The drug 3-nitro was not included in the wide variety of arsenicals used in tests reported in the literature in which a protective effect was obtained from PABA. However, *N*-carbamylarsanilic acid has been studied extensively. We conducted one experiment with chicks to determine if prior administration of 1,000 mg. of PABA per kilogram of body weight could meliorate a dose of 1,000 mg. of *N*-carbamylarsanilic acid. That this level was not toxic was shown by the lack of mortality. All birds gained weight, averaging 45.6 Gm. and 46.2 Gm. for the combination and the *N*-carbamylarsanilic acid alone, respectively.

POULT STUDIES

Experiment 1.—The results of the two poult experiments are shown (table 3). In the first experiment, levels of 20 and 50 mg. of 3-nitro or PABA per kilogram of body weight, or both, were used. At the lower level, either in the presence or absence of PABA, there was no mortality and all poults made increases in weight during the five-day observation period.

At the higher level, some individuals lost weight; however, the average gains of survivors in these groups were 86.9 Gm. and 68.0 Gm. for the 3-nitro alone and 3-nitro plus PABA, respectively. One out of 10 poults died in the absence of PABA and 3 out of 10 in the presence of PABA.

Experiment 2.—In the second experiment with poults (table 3), the only mortality was in the group given 80 mg. of 3-nitro preceded by PABA. In this group, 2 of the 10 birds died, although the survivors gained more weight than did those given the 80-mg. level of 3-nitro without PABA. Administration of sodium bicarbonate solution alone had no adverse influence. The 20-mg. level of 3-nitro, with and without PABA, had little influence on weight and on growth. Growth was less at the 40-mg. level of 3-nitro; the prior dose of PABA may have increased this adverse effect.

The greater gains made by the lot given sodium bicarbonate solution alone, as compared with the control, is probably without significance, since the more vigorous birds were used in nine of the experimental groups and the lighter birds, and apparently growing at a less rapid rate, were used as controls.

When the gains of all poults given the 20-mg. per kilogram level of 3-nitro (sepa-

TABLE 3—Effect of PABA and Sodium Bicarbonate on Weight Gains and Livability of Poults Given 3-Nitro-4-Hydroxyphenylarsonic Acid (Experiments 1 and 2)

3-nitro (mg./kg.)	PABA (mg./kg.)					Bicarbonate solution	
	0	20	40	50	80	40	80
0	*309.7(10)	.....	.....	.....	.....	*342.0(10)	.....
20	231.8(10)	201.2(10)	.....	.....	.....	.....	.....
	*296.5(10)	*273.2(10)	.....	.....	.....	.....	.....
40	*264.0(10)	.....	*245.2(10)	.....	.....	*270.6(10)	.....
50	86.9(9)	.....	.....	68.0(7)	.....	.....	.....
80	*74.2(10)	.....	.....	.....	*123.9(8)	.....	*11.2(10)

Average weight gain is given in grams. The figure in parentheses represents the number of survivors out of 10 treated poults.

* Experiment 2.

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rate experiments treated as replications) were evaluated by analysis of variance, the differences in gains were not statistically significant.

DISCUSSION

Frost and Spruth [2] reported the largest single dose of 3-nitro tolerated by Beltsville Small White turkeys to be 20 mg. per kilogram of body weight. Our results show Broad-Breasted Bronze poults tolerate a single dose of at least 40 mg. per kilogram of body weight with 80 mg. per kilogram constituting less than a $l.d._{10}$.

Neither chicks nor poults exhibited tolerance to 3-nitro, given as a single dose following administration of PABA, as has been reported for rats given pentavalent organic arsenicals. Prior administration of PABA makes an otherwise $l.d._{90}$ of many pentavalent organic arsenicals nonlethal to rats. Prior administration of PABA to chicks given 200 mg. of 3-nitro per kilogram of body weight decreased mortality from 98 to 83 per cent. This was a highly significant difference, but relatively ineffective when compared with the protective effect obtained in rats.

It has been suggested by Martin [3] that PABA lowers the metabolic rate and reduces the speed with which pentavalent arsenicals are reduced to the trivalent, more toxic, arsonoxide. If this theory is true, one would expect poultry to be less tolerant of organic arsenicals than rats or swine, which have a lower metabolic rate. According to the report of Frost and Spruth,[2] such a difference does not exist. Furthermore, if PABA mediates its detoxifying effect by depressing thyroid activity, one would expect a more dramatic effect from PABA in chicks than in rats. This was not the case. The data obtained indicate that a species difference exists between rats and poultry in their ability to benefit from a prior dose of PABA when given a single toxic dose of a pentavalent organic arsenical.

SUMMARY

In three experiments, 210 chicks, 4 weeks of age or older, were used to determine if the prior administration of a quantity of para-aminobenzoic acid (PABA), equivalent in weight or exceeding that in a single dose of 3-nitro-4-hydroxyphenylarsonic acid (3-nitro), would protect against intoxication. The PABA had no effect on mortality at the 100-mg. per kilogram level of 3-nitro, although it appeared to increase survival time. The mortality when PABA was given was 44.4 per cent and, in the absence of PABA, 36 per cent (a nonsignificant difference). At the 200-mg. level, there was a highly significant difference due to the prior administration of PABA.

Administration of *N*-carbamylarsanilic acid at a level of 1,000 mg. per kilogram of body weight was not lethal, and prior treatment with PABA had no effect on weight gains during the observation period.

In tests using 140 poults given levels of 20, 40, 50, and 80 mg. of 3-nitro per kilogram of body weight, the prior administration of equivalent quantities of PABA was without effect. A level in excess of 40 mg. per kilogram of body weight of 3-nitro was required before mortality occurred. Of the 20 birds treated at the 50-mg. level, 4 died; of the 30 treated at the 80-mg. level, 2 died.

References

[1] Bird, H. R., Almquist, J. H., Cravens, W. W., Hill, F. W., and McGinnis, J.: Nutrient Requirements for Poultry. National Research Council Pub., 301, 1954.

[2] Frost, D. V., and Spruth, H. C.: Arsenicals in Feeds. In "Symposium on Medicated Feeds." New York Medical Encyclopedia, Inc., (1956): 136.

[3] Martin, G. J.: The Detoxification of Pentavalent Arsenicals. Am. J. Pharmacol., *118*, (1946): 394.

[4] Milligan, J. L., Wilcke, H. L., Marr, J. E., and Bethke, R. M.: Arsonic Acid in Commercial Broiler Rations. Poult. Sci., *34*, (1955): 794.

[5] Morehouse, N. F., and Mayfield, O. J.: The Effect of Some Arylarsonic Acids on Experimental Coccidiosis Infection in Chickens. J. Parasitol., *32*, (1946): 20.

[6] Moshkovskii, Sh. d., and Stoyanova, A. V.: Inhibitory Effect of Para-Aminobenzoic Acid and Sulfanilamide on the Chemotherapeutic Action of the Aromatic Derivatives of Arson. Byull. Eksptl. Biol. Med., *23*, (1947): 202. (Chem. Abstr., 42, [1948] 2672F).

[7] Sandground, J. H.: Studies on the Detoxification of Organic Arsenical Compounds. III. The Time Factor Influences Para-Aminobenzoate Protection of Rats Receiving Lethal Doses of Phenyl Arsonates. J. Pharmacol. & Exptl. Therap., *78*, (1943): 209.

[8] Sandground, J. H., and Hamilton, C. R.: Studies on the Detoxification of Organic Arsenical Compounds. I. Detoxification by Means of Para-Aminobenzoic Acid of Certain Pentavalent Arsenical Drugs Given in Massive Doses to Rats. J. Pharmacol. & Exptl. Therap., *78*, (1943): 109.

[9] Sandground, J. H., and Hamilton, C. R.: Studies on the Detoxification of Organic Arsenical Compounds. IV. The Protective Action of Para-Aminobenzoic Acid Against Lethal Doses of Nelarsphenamine Without Inhibition of Trypanocidal Potency. J. Lab. & Clin. Med., *28*, (1943): 1821.

[10] Sandground, J. H., and Hamilton, C. R.: Studies on the Detoxification of Organic Arsenical Compounds. II. Correlation of the Quantity of Para-Aminobenzoic Acid Required to Protect Rats Against High Doses of Carbarsone and Arsanilic Acid. J. Pharmacol. & Exptl. Therap., *78*, (1943): 203.

[11] Snedecor, G. W.: Statistical Methods. Iowa State College Press, Ames (1946): 194.

[12] Williamson, J., and Lourie, E. M.: Interference with the Trypanocidal Action of *gamma* (p-arsenosophenyl) Butyric Acid by Para-Aminobenzoic Acid. Ann. Trop. Med. & Parasitol., *40*, (1946): 255.

The Treatment of *Salmonella Gallinarum* Infection in Turkey Poults with Chloramphenicol and Furazolidone

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THE ANTIBACTERIAL PROPERTIES of furazolidone (Furoxone,* or N-[5-nitro-2-furfurylidene]-3-amino-2-oxazolidone) were first reported in 1953.[8] It has since found wide use in the prevention and treatment of salmonelloses in poultry.[3-6] Chloramphenicol (Chloromycetin†), when given by capsule or in mash, was found to be the most effective of seven antibiotics used in a study of experimental fowl typhoid.[2] This report presents further results with chloramphenicol and furazolidone in turkey poults artificially infected with *Salmonella gallinarum*.

EXPERIMENTAL PROCEDURE

Broad-Breasted Bronze poults known to be free of *S. gallinarum* infection were obtained. They were allotted randomly to groups of 10 birds each and housed throughout the experiment in wire-floored batteries, with the noninoculated groups kept separate. At 10 days of age, the birds were inoculated intramuscularly with 10^6 viable *S. gallinarum* from broth culture of proved virulence. Including controls, eight groups were used in the chloramphenicol study and nine groups in the furazolidone study. Treatment with furazolidone (0.011 or 0.022% in the feed) or chloramphenicol (0.22% in the feed, or 50-mg. capsules) was begun at various times before or after inoculation and continued for three to 17 days in various groups to permit evaluation of the two drugs in preventing immediate deaths and also in protecting against later exacerbations of infection.

The observation period was extended for 25 days after the end of treatment to detect development of the carrier state or delayed signs of infection. At the end of the observation period, whole blood plate-agglutination tests were run on surviving birds with a standard pullorum-plate antigen.

RESULTS AND DISCUSSION

The research outline and results are shown (table 1). Although mortality among the untreated controls was 100 per cent, no deaths occurred during the first four days after inoculation. All deaths in treated groups occurred only after treatment was instituted. Preinoculation treatment with either furazolidone (0.011% or 0.022%) or chloramphenicol at 0.22 per cent prevented mortality from fowl typhoid for 12 days postinoculation; however, complete group protection did not extend for 37 days. Except for poults given furazolidone at 0.022 per cent, surviviors reacted strongly to the plate agglutination test (table 1, pens 1, 2, 7).

When furazolidone was started on the day of inoculation and fed continuously for 12 days, the drug failed to prevent deaths during this period. At the close of the observation period most survivors were disease carriers (table 1, pens 3 and 4). However, resistance to infection appeared to have developed for these groups, as no deaths occurred after medication was discontinued.

Treatment starting on the fourth day postinoculation gave poor protection against fowl typhoid. Of 10 poults fed 0.22 per cent chloramphenicol for eight days, 8 died within 12 days after *S. gallinarum* inoculation. Of 10 poults fed chloramphenicol at 50 mg. per day for three days, 6 died; of 10 poults fed furazolidone (0.011%) for eight days, 5 died, all within the same 12-day postinoculation period. However, only 1 of 10 poults succumbed after treatment with 0.022 per cent furazolidone for eight days beginning four days postinoculation. Twenty-five days later in this group, there had been only 1 other fatality, although 5 of the 8 survivors reacted positively to the agglutination test. There was no sign of drug toxicosis or other untoward reaction in birds of any group given either furazolidone or chloramphenicol.

The superiority of furazolidone is probably due to its bactericidal properties, as contrasted with the bacteriostatic action of chloramphenicol.[6] Rapid destruction of the organisms by furazolidone could conceiva-

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* Furoxone, furnished by Eaton Laboratories, Norwich, N.Y. † Chloromycetin, furnished by Parke-Davis and Co., Detroit, Mich.

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bly account for the lack of agglutinin development and the suppression of the agglutination reaction. This information should be of considerable practical value because the young poult is most susceptible to Salmonella infections and infected flocks are rarely treated before signs of the disease appear.

The number of furazolidone-treated birds which become free of infection (nonreactors) and birds which become possible carriers (reactors) is of interest. Breeding-flock owners and hatcherymen would like to know if treatment of such infections in their flocks is correct and economically sound in regard to the pullorum-testing program.

Chicken or turkey flocks infected with fowl typhoid organisms should not be used for breeding. Elimination of infected birds through testing and slaughter of birds that react to an adequately sensitive test after treatment would seem necessary to obtain a clean flock. In an outbreak of salmonellosis, birds in all stages of infection would be found. If such a flock were treated early in the course of the disease, it is conceivable that some birds would not produce sufficient agglutinins to give a positive reaction. Many of these birds, being retained in the breeding flock, might relapse and spread the infection.

Other work [6] indicates that a policy of eradication based on agglutination tests initiated too soon after the completion of high-level treatment (10 days after recovery) might result in the elimination of birds that were no longer infected. It was found that many chickens treated ten days after infection had developed agglutinins with a positive reaction to the plate test but were negative on culture. These same birds became agglutination-negative after a short time. Similar changes from positive to negative have been reported with sulfamerazine in pullorum-infected hens.[1]

In more recent studies [7] with furazolidone in hens naturally infected with pullorum disease, the drug failed to alter the serum agglutination titers, and *S. pullorum* could be recovered from the birds giving a positive test. It was also found that in artificially infected birds, medication greatly reduced the infectivity of the organism, as shown also by lack of agglutinin production in the majority of treated chickens.[7]

These results and observations indicate that furazolidone has a definite place in treatment of fowl typhoid in poults. However, more information is needed in regard to the carrier status and agglutination reaction. The answer to some of these questions may depend upon close observation of (1) the type of Salmonella infection present in the flock, (2) the time of treatment in regard to infection, and (3) the virulence of the infecting organisms.

SUMMARY

Turkeys, 10 days old, were artificially infected with approximately 10^6 *Salmonella gallinarum* cells. While mortality in the untreated control group was 100 per cent, death losses were prevented by feeding chloramphenicol at 0.22 per cent or furazoli-

TABLE 1—Results with Furazolidone and Chloramphenicol in 10-Day-Old Turkey Poults Inoculated with Salmonella Gallinarum

		Treatment*				Results		
Pen No.	No. birds	Furazolidone dosage	Chloramphenicol dosage	Start (days)	Duration (days)	Dead/inoc.	Relapses† Dead/inoc.	+ Agglut./ survivors
1	10	0.011%	.....	5 preinoc.	17	0/10	3/10	5/7
2	10	0.022%	.....	5 preinoc.	17	0/10	2/10	2/8
3	10	0.011%	.....	Day of inoc.	12	4/10	0/10	4/6
4	10	0.022%	.....	Day of inoc.	12	2/10	0/10	5/8
5	10	0.011%	.....	4 postinoc.	8	5/10	3/10	2/2
6	10	0.022%	.....	4 postinoc.	8	1/10	1/10	5/8
7	10	.....	0.22%	5 preinoc.	17	0/10	6/10	4/4
8	10	.....	0.22%	Day of inoc.	12	3/10	6/10	1/1
9	10	.....	50 mg./day	Day of inoc.	3	2/10	5/10	2/3
10	10	.....	0.22%	4 postinoc.	8	8/10	1/10	1/1
11	10	.....	50 mg./day	4 postinoc.	3	6/10	2/10	2/2
12	10	0.011%	.....	uninoc.-cont.	17	.....	.....	0/10
13	10	0.022%	.....	uninoc.-cont.	17	.....	.....	0/10
14	10	.....	0.22%	uninoc.-cont.	17	.....	.....	0/10
15	10	.....	50 mg./day	uninoc.-cont.	3	.....	.....	0/10
16	10	.....	.....	inoc.-cont.	....	10/10	.....	.....

* Drugs incorporated in ration except 50 mg./day chloramphenicol capsules given *per os*. † Mortality occurring 12 to 37 days after inoculation.

done at 0.011 per cent or 0.022 per cent in feed starting five days before inoculation and continuing for 17 days.

When treatment was begun at the time of infection and continued for 12 days, deaths occurred in all groups. When medication was started four days after inoculation and continued for eight days, more furazolidone- than chloramphenicol-medicated birds survived. Chloramphenicol given at the rate of one 50-mg. capsule daily for three days was of little value in preventing deaths when given either at the time of inoculation or at appearance of illness four days later.

Agglutination tests were negative in only 1 of the survivors medicated with chloramphenicol, but were negative in many of the survivors medicated with furazolidone.

References

[1] Cooper, J. B., Morgan, C. L., Anderson, G. W., and Jones, J. C.: Effect of Sulfamerazine on Pullorum Reactors. Poult. Sci., *30*, (1951): 249–254.

[2] Glantz, P. J., and Gordeuk, S.: *In Vitro* and *in Vivo* Sensitivity of the Fowl Typhoid Organisms, *Salmonella Gallinarum*, to Antibiotics. Poult. Sci., *34*, (1955): 880–890.

[3] Grumbles, L. C., Wills, F. K., and Boney, W. A.: Furazolidone in the Treatment of Fowl Typhoid in Turkeys. J.A.V.M.A., *124*, (March, 1954): 217–219.

[4] Harwood, P. D., and Stunz, D. L.: The Treatment of Fowl Typhoid with Furazolidone. Proc. 10th World's Poultry Congress, Edinburgh, (1954): 222–224.

[5] Smith, H. W.: The Treatment of *Salmonella Pullorum* Infection in Chicks with Furazolidone, Sulphamerazine, and Chloramphenicol. Vet. Rec., *66*, (1954): 493–496.

[6] Smith, H. W.: The Chemotherapy of Experimental Fowl Typhoid in Fowls *(Gallus Domesticus)*. J. Comp. Path. & Therap., *65*, (1955): 55–70.

[7] Smyser, C. F., and Van Roekel, H.: The Influence of Furazolidone on Experimental and Natural Pullorum Infection in the Chicken. Avian Dis., *1*, (1957): 328–337.

[8] Yurchenco, J. A., Yurchenco, M. C., and Piepoli, C. R.: Antimicrobial Properties of Furoxone (N-5-Nitro-2-Furfurylidene-3-Amino-2-Oxazolidone). Antibiotics, *3*, (1953): 1035–1039.

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Anthelmintic Studies with Carbon 14-Labeled Carbon Tetrachloride on *Ascaridia Galli* (Nematoda) and Its Chicken Host

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DESPITE THE INTRODUCTION of several new anthelmintics effective against adult *Ascaridia galli*, several recent investigators, including Hammond [4] in Kenya, Semikin [10] along with Akhumgan and Oganyon [1] in Russia, Martin [5] in Spain, and Mohan [8] in India, have recommended the use of carbon tetrachloride (CCl_4) for the control of this parasite. No work has been reported on either the uptake of CCl_4 by this roundworm or the distribution of this compound in the chicken host.

The purpose of the present study was to determine the uptake of carbon 14-labeled CCl_4 in *A. galli* and its host after a therapeutic dose of the compound.

MATERIALS AND METHODS

An infected White Plymouth Rock cockerel, weighing 1,160 Gm., was given 1.1 ml. of $C^{14}Cl_4$ administered in two gelatin capsules. Terhaar [12] showed that this amount of CCl_4 has approximately a 75 per cent efficacy. Enough $C^{14}Cl_4$ (specific activity of 3,250 disintegrations $sec.^{-1}$ $mg.^{-1}$) was available to treat 1 bird. After treatment, the bird was placed in an oilcloth-lined wire cage and kept under constant observation. The feces, urine, and expelled worms were collected after each defecation; no attempt was made to collect and measure expired C^{14}-labeled compounds.

Each expelled worm was rinsed in inactive CCl_4 and quick-frozen in dry ice, as were all excreta. The bird was killed 6.4 hours post-treatment; three samples of various tissues were removed, weighed (av., about 60 mg.), and quick-frozen. All worms were expelled by 6.4 hours post-treatment, since no worms were recovered at necropsy.

The worms and tissue samples were oxidized in 5 ml. of Van Slyke oxidizing fluid (Comar[2]) and the evolved CO_2 and $C^{14}O_2$ were absorbed in a NaOH solution. The NaOH solution containing the trapped CO_2 was transferred to an apparatus described by Comar,[2] where it was acidified with perchloric acid. The contents were boiled and the evolved CO_2 and $C^{14}O_2$ were swept out through a sintered glass-mercury seal into an evacuated 250-ml. ionization chamber. The disintegration rate, as a function of ionization current, was determined with a vibrating reed electrometer.* The C^{14} measurement was used to calculate the quantity of CCl_4 needed to produce an equivalent counting rate. The chemical form of the C^{14} was not determined in the present study (throughout this paper the expression "C^{14}" refers to C^{14}-labeled compounds; no qualitative analyses were made on the C^{14}-containing samples).

No correction was made for decay of C^{14}, since its half-life is in excess of 5,000 years.

RESULTS

The results show that all organs of the host chicken studied contained C^{14} (table 1). The ceca contained the most radioactivity, and the testes contained the least.

The total amount of blood in the bird was calculated on the basis of Sturkie's [11] report that the blood volume (ml.) of a bird of this weight is equal to 9 per cent of the total body weight (Gm.). Since he also showed that the specific gravity of chicken blood is 1.05, the weight of the blood equals the total body weight times 9 times 1.05. The value for the bone weight was calculated on the basis that bone accounts for about 17 per cent of the total carcass weight after the feet and head are removed.[7] Ewing [3] reported that the head and feet accounted for about 9 per cent of the total body weight of a chicken.

The C^{14} (expressed as CCl_4) values given

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Based in part on a thesis submitted by the senior author in partial fulfillment of the requirements for the Ph.D. (parasitology) degree.

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* Vibrating Reed Electrometer Model 30M, manufactured by Applied Physics Corp., Pasadena, Calif.

for muscle were based on muscle tissue samples. The total weight of the muscle tissue was not known and was actually the residual weight of the bird (the weight obtained by adding the actual organ, excreta, and calculated bone and blood weights and subtracting from the total live weight).

The C^{14} appeared in the excreta 15 minutes post-treatment and increased in amounts over the period studied (table 2).

No relationship between either the sex or weight of worms and CCl_4 uptake was indicated (table 3). However, the small number of worms found precluded any definite conclusion as to uptake of CCl_4 relative to sex and weight of worms. The selective action of CCl_4 on *A. galli* was evident when uptake of C^{14} by the worms was compared with the uptake by the host body. The worms averaged 62.6 mg. of CCl_4/100 Gm. of worms, calculated from the total C^{14}. The highest concentration of C^{14} in the host's body was in the ceca, which averaged 6.9 mg. of CCl_4/100 Gm. of tissue.

TABLE 1—Distribution of C^{14}, Expressed as CCl_4, in the Chicken

Organ	Organ wt. (Gm.)	Total CCl_4 in organ (mg.)	Mg. CCl_4/100 Gm. of tissue
Duodenum	19.8	1.34	6.7
Lower small intestine	20.7	0.78	3.8
Ceca	15.0	1.04	6.9
Colon	9.5	0.28	2.9
Liver	28.4	0.82	2.9
Blood	109.6	2.71	2.4
Bone	179.5	3.89	2.2
Spleen	1.9	0.04	2.1
Heart	5.9	0.10	1.7
Lungs	8.3	0.07	0.8
Muscles	718.1	6.10	0.8
Testes	1.8	0.01	0.6

DISCUSSION

The C^{14} which remained in the host's tissues at necropsy probably represents bound or metabolized CCL_4, or both. In repeated trials, using a colorimetric method which could detect as little as 8 μg. of CCl_4, no free CCl_4 was found in the tissues of treated birds or expelled worms. However, the C^{14} concentrations, expressed as CCl_4, were higher in certain tissues and in most of the worms than the lower limit of detection with the colorimetric system. This would indicate that a large percentage of the C^{14} was actually not in the tetrachlorinated form.

Earlier work by McCollister *et al.*[6] and Robbins[9] has shown that practically all of the CCl_4 absorbed by an animal is excreted through the lungs. This is apparently true in the present study, since relatively minute amounts were recovered from the organs and excreta.

TABLE 2—Distribution of C^{14}, Expressed as CCl_4, in Excreta of a Chicken

Time post-treatment (hr.)	Weight of excreta (Gm.)	Total CCl_4 (mg.)	Mg. CCl_4/100 Gm. of excreta
0.25	12.53	0.17	1.4
2.25	14.69	0.40	2.7
6.41	15.43	2.61	16.9

It seems reasonable that the administered CCl_4 passed rather quickly into the gizzard and duodenum. Most of the CCl_4 was probably absorbed into the capillaries and lacteals of the duodenum and anterior jejunum, eventually reaching the lungs to be excreted. Of the fraction that was not excreted by the lungs, a substantial portion was probably excreted into the ceca from which it passed to the cloaca and was eliminated.

Evidence for assuming cecal excretion of CCl_4 is supplied by the fact that, 15 minutes after treatment, C^{14} was recovered in the feces. It is unlikely that CCl_4 could pass the entire length of the digestive tract in this short time. The earliest period at which either oil red O in CCl_4, or powdered fluorescent minerals reached the large intestine was 3.5 hours.[12] Collaborative evidence for assuming absorption in the duodenum and excretion in the ceca is provided by the fact that the ceca had the highest concentrations of C^{14} per unit weight of organ; the duodenum had the next highest concentration. The part of the digestive system between the duodenum

TABLE 3—Distribution of C^{14}, Expressed as CCl_4, in Expelled Ascaridia galli

Worm No.	Sex	Weight (mg.)	Total CCl_4 (mg.)	Mg. CCl_4/100 Gm. of worms
1	F	72	0.006	8.7
2	M	37	0.016	42.9
3	F	69	0.014	20.0
4	F	25	0.006	22.7
5	M	32	0.022	68.6
6	M	32	0.007	23.3
7	M	34	0.028	82.5
8	F	62	0.062	99.4
9	F	47	0.032	67.9
10	F	60	0.036	60.3
11	M	40	0.076	190.6

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and ceca had considerably less C^{14} present, indicating that a large amount of CCl_4 never passed the entire length of the digestive tract.

The CCl_4 that was not absorbed into the circulatory system presumably passed through the digestive tract, and was eliminated and counted with the feces and urine. As it passed through the gut, it probably immobilized and killed the worms, which then lost their power to resist the fecal stream. This would explain the observation that expelled worms are generally found in a formed stool.

Unfortunately, no samples of kidney tissue were carried through the entire analytical procedure. However, from the results of McCollister *et al.*,[6] and from a comparison of the relative amounts of C^{14} found in the ceca and excreta in the present study, it may be assumed that relatively little C^{14} was excreted in the urine.

The apparent extreme difference in C^{14} uptake per milligram of worms between worms 1 and 11 (table 3) actually amounted to only 0.044 μl. of CCl_4. Extremely little is known concerning the feeding habits of *A. galli* in the digestive tract. If the worms feed passively, that is, allow the stream of chyme to flow through them at a more or less constant rate, then the amounts of CCl_4 present should be a function of the length of the digestive tract. If, however, the worms feed actively but not constantly, it can be reasoned that the amount of CCl_4 in the digestive tract could easily fluctuate within the range determined.

SUMMARY

Carbon 14-labeled carbon tetrachloride was administered to a chicken harboring *Ascaridia galli*. The results of the subsequent analyses showed that C^{14} was present in the tissues and excreta of the host as well as in the worms. In the host, the greatest uptake of C^{14} was in the ceca and the least in the testes. Carbon-14 appeared in the excreta 15 minutes after treatment. The average uptake by the worms was much greater than that of any of the tissues in the host.

References

[1] Akhumgan, K. S., and Oganyon, M. G.: A New Simplified Method Treating Domestic Birds with Carbon Tetrachloride. Veterinariya, *28*, (1951): 31.

[2] Comar, C. L.: Radioisotopes in Biology and Agriculture. McGraw-Hill Book Co., New York, N.Y., 1955.

[3] Ewing, W. R.: Poultry Nutrition. 4th ed. W. R. Ewing, South Pasadena, Calif., 1951.

[4] Hammond, R. A.: Report of the Kenya Colony Veterinary Service, Gen. Zool. Helminths, (1954): 159–61.

[5] Martin, V. B.: Ascaridiosis de las Gallinas *Ascaridia Galli*, Fenctiazina y Tetracloruro de Carbono. Veterinaria, *16*, (1952): 807–814.

[6] McCollister, D. D., Beamer, W. H., Atchison, G. J., and Spencer, H. C.: The Absorption, Distribution and Elimination of Radioactive Carbon Tetrachloride by Monkeys Upon Exposure to Low Vapor Concentrations. J. Pharmacol. & Exptl. Therap., *102*, (1951): 112–124.

[7] Mitchell, H. H., Card, L. E., and Hamilton, T. S.: The Growth of White Plymouth Rock Chickens. University of Illinois Agric. Exper. Sta. Bull. 278, 1926.

[8] Mohan, R. N.: Treatment of *Ascaridia Galli* in Poultry with (a) Carbon Tetrachloride and (b) Oil of Chenopodium. Indian Vet. J., *30*, (1954): 412–417.

[9] Robbins, B. H.: The Absorption, Distribution and Excretion of Carbon Tetrachloride in Dogs Under Various Conditions. J. Pharmacol. Exptl. Therap., *37*, (1929): 203–216.

[10] Semikin, V. I.: Treatment of Ascaridiasis in Chickens. Veterinariya, *29*, (1952): 43.

[11] Sturkie, P. D.: Avian Physiology. Comstock Publishing Associates, Ithaca, N.Y., 1954.

[12] Terhaar, C. J.: Carbon Tetrachloride as an Anthelmintic: Its Effect on and Distribution in a Parasite (*Ascaridia Galli*) and Its Host. Ph.D. dissertation (unpublished), Kansas State College, Manhattan, 1957.

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Virucidal Effect of Some Chemical Agents on Bluetongue Virus

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IN AN EXTENSIVE review of the literature pertaining to bluetongue in sheep, nothing was found to indicate that any studies had been made to determine the virucidal effect of chemical agents on the causative virus. Our primary interest was to find if one of the chemical agents frequently used for disinfectant purposes would be effective as a rapidly acting virucide against the virus of bluetongue which was (1) adapted in embryonating chicken eggs and (2) present in serum collected during the early febrile period from a sheep reacting to an injection of bluetongue virus.

To determine this, five chemicals were selected for the tests, which involved the use of chicken embryos and sheep for evaluating the effectiveness of the chemical solutions as virucides.

MATERIALS AND METHODS

Isolation and Identification of the Virus.—A single strain of bluetongue virus, designated as New Mexico BT-52, was used in these experiments. It was isolated in October, 1955, in sheep by the passage of pooled blood samples, collected in an equal volume of O.C.G.* solution,[3] from 2 sheep during a natural outbreak of bluetongue in a flock of sheep in New Mexico.

The virus was identified by reciprocal cross-immunity tests in sheep using the known bluetongue virus strain, California BT-11,[2] as the standard. Each group of test sheep was housed in a separate insect-proof isolation room until the homologous challenge test of their immunity was completed. The animals were then transferred by cross exchange from one isolation room to the other and cross challenged. The virus isolates were considered to be immunologically identical, since no febrile reaction or clinical signs of bluetongue were manifested. The sheep that were used for virus controls in each test to confirm the infectivity of the virus developed signs of clinical bluetongue. The sheep which served as contact controls remained normal. Temperatures of all animals were taken twice daily.

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* An anticoagulant preservative solution consisting of potassium oxalate, 5 Gm.; phenol, 5 Gm.; glycerin, 500 ml.; and distilled water, 500 ml.

TESTS USING EMBRYONATING CHICKEN EGGS

Incubation and Inoculation of Eggs.—Embryonating chicken eggs that had been preincubated for seven or eight days at 37.5 C. in an electric forced-draft incubator were used in all the tests. The shell over the air sac was disinfected with Lugol's solution of iodine. A small opening was made in the shell with an electric vibrating drill (Vibro-Tool†). Injections of the inoculum were made via the yolk sac route with a 20- or 23-gauge, 1-inch needle, depending upon the size of the particles in the inoculum used. The opening in the shell was closed with sealing wax, and the eggs were placed in the incubator and incubated at 33.5 C.[1] for seven days. The eggs were candled twice daily.

Propagation of the Virus.—The propagation of the virus was established by serial passage inoculations via the yolk sac route in embryonating chicken eggs as described. All embryos dying within 24 hours after injection were discarded. Embryos dying on the second and third day post-inoculation were harvested for virus. The virus used was from the thirty-second or subsequent passages in embryos.

Preparation of the Virus.—Two or three embryos were ground at a time without diluent in a TenBroeck grinder, cultured to determine that they were free of bacterial contaminants, and stored in screw-capped tubes at 4 C. until needed. The three virus preparations used for inoculums were (1) undiluted ground chicken embryos; (2) undiluted chicken embryo supernatant fluid, obtained by centrifuging the ground embryos for 15 minutes at 1,500 r.p.m. at 5 C., decanting the fluid, and clarifying it by another centrifugation; and (3) chicken embryo supernatant fluid diluted 1:9 with beef-heart infusion broth (Difco) at a pH of 7.2. For each test, bacteriological cultures were made before the virus suspensions were used to ensure their freedom from bacterial contamination.

Titration of the Virus.—The virus was titrated in each test, using either beef-heart infusion broth at a pH of 7.2 or buffered saline solution at a pH of 7.2 as the diluent. One tenth milliliter of the tenfold dilutions of inoculum were injected into the yolk sac of 7- to 8-day embryonating eggs. Ten eggs were used per dilution. The 50 per cent embryo lethal dose ($e.l.d._{50}$) titer of the virus was calculated by the method of Reed and Muench.[4]

† Vibro-Tool is manufactured by Burgess Vibrocrafters, Inc., Grayslake, Ill.

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Chemical Agents.—The chemical agents selected for study in the embryonating egg tests were Roccal * 50 per cent (hereafter referred to as agent R); Wescodyne † (referred to as agent W); sodium hydroxide; and sodium carbonate.

Virus and Chemical Inoculum.—A fresh solution of the chemical agent was prepared at the beginning of each test. Each chemical agent was diluted with sterile tap water to make twice the desired percentage or parts per million (p.p.m.) concentration. Equal volumes of the virus suspension and chemical solution were dispensed in a screw-capped tube, thoroughly mixed, and held at 22 C. Between the four- and the six-minute reaction period, each of 18 to 28 embryonating eggs that had been preincubated for seven to eight days was inoculated via the yolk sac route with 0.2 ml. of the selected virus and chemical inoculum.

Chemical Agent and Diluent Control.—In each experiment, chemical controls were run by mixing equal volumes of the chemical solution and beef-heart infusion broth at a pH of 7.2 or buffered saline solution at a pH of 7.2, depending upon which was used as the dilutent for the virus.

Incubator Control.—In each test, noninoculated 7- or 8-day embryonating eggs were placed in the incubator with the inoculated eggs to serve as incubator controls.

Embryo Deaths Caused by Virus.—A number of tests were run to determine the presence of virus

* Roccal (alkyldimethylbenzyl ammonium chloride) is produced by Winthrop Laboratories, New York, N.Y.

† Wescodyne (polyethoxypolypropoxypolyethoxy glycol-iodine complex, 7.75%; nonyl phenylether of polyethylene glycol-iodine complex, 7.75%; hydrogen chloride, 0.1% [providing 1.6% available iodine]; and inert material, 84.4%) is produced by West Chemical Products Inc., Long Island, N.Y.

TABLE 1—Effect of Exposure to Chemical Agents of Undiluted Ground Chicken Embryo Infected with Bluetongue Virus

Chemical agent	Final chemical concentration	Virus titer	Results of injection of eggs with virus chemical and chemical diluent previously incubated for 4 to 6 min. at 22 C.		Virus chemical subpassage; embryo deaths
			Virus chemical	Chemical diluent (controls)	
		B.H.I.B.* diluent		B.H.I.B.*	
Agent R	5,000 p.p.m.	$10^{3.9}$†	13/15**	4/21	.....
	5,000 p.p.m.	$10^{3.7}$	10/18	2/19	.....
Agent W	1,000 p.p.m.	$10^{4.4}$	1/12	2/15	0/10
	1,500 p.p.m.	$10^{4.4}$	3/13	2/15	0/10
Sodium hydroxide	2.0%	$10^{4.4}$	6/13	1/17	.....
	3.0%	$10^{4.4}$	1/14	0/13	0/11
		Buffered saline diluent		Buffered saline solution	
Agent R	5,000 p.p.m.	$10^{4.5}$	12/15	0/18	6/7
Agent W	500 p.p.m.	$10^{4.0}$	8/26	1/18	14/16
	500 p.p.m.	$10^{4.4}$	10/28	0/15	.....
	500 p.p.m.	$10^{4.5}$	12/21	0/20	18/18
	750 p.p.m.	$10^{4.5}$	1/17	0/18	0/6
	750 p.p.m.	$10^{4.1}$	1/16	0/12	0/12
	750 p.p.m.	$10^{4.3}$	2/14	3/14	0/9
	750 p.p.m.	$10^{4.0}$	1/25	1/19	0/10
	750 p.p.m.	$10^{4.4}$	0/26	0/17	.....
	750 p.p.m.	$10^{4.5}$	1/21	0/22	0/6
	1,000 p.p.m.	$10^{4.5}$	2/20	0/15	0/9
	1,000 p.p.m.	$10^{4.1}$	2/14	2/10	0/9
	1,000 p.p.m.	$10^{4.3}$	0/14	2/13	.....
Sodium hydroxide	2.0%	$10^{4.5}$	3/13	5/17	7/7
	2.0%	$10^{4.1}$	5/16	0/11	9/9
	2.0%	$10^{4.3}$	8/16	4/10	11/11
	2.5%	$10^{4.0}$	8/25	1/16	10/11
	2.5%	$10^{4.4}$	5/28	1/19	.....
	2.5%	$10^{4.5}$	8/21	1/16	9/9
	3.0%	$10^{4.5}$	0/10	1/12	.....
	3.0%	$10^{4.1}$	0/14	0/11	.....
	3.0%	$10^{4.3}$	3/13	1/9	0/9
	3.0%	$10^{4.0}$	0/12	0/19	.....
	3.0%	$10^{4.4}$	5/22	2/14	7/8
	3.0%	$10^{4.5}$	5/21	1/21	9/9

* B.H.I.B. = Beef-heart infusion broth (Difco), pH 7.2. † The virus titer is expressed as the chicken embryo l.d.$_{50}$ per 0.1 ml. ** The numerator indicates the number of embryos dying from the second through the seventh day, and the denominator represents the number of eggs injected after discarding those containing embryos which died within the first 24 hours of injection.

in embryos that died after inoculation with the virus-chemical inoculum.

Test Using Sheep

Selection, Identification, and Housing of Sheep.—The sheep used in the experiments were from a flock susceptible to bluetongue and maintained at the research station. They were housed in insect-proof isolation rooms and identified by ear-tag numbers. Temperatures were taken twice daily during the course of the tests.

Source of Virus.—The virus material used was New Mexico BT-52-infected sheep serum (fifth virus passage in sheep).

Chemical Agents.—The chemicals selected for these tests were agent W, sodium hydroxide, and ethyl alcohol.

Virus and Chemical Inoculum.—A fresh solution of the chemical was prepared at the beginning of each test, using sterile tap water for the diluent. Agent W, at a concentration of 1,500 p.p.m., was added to an equal volume of virus-infected sheep serum in a screw-capped test tube and thoroughly mixed. After a five-minute reaction period at room temperature, 8 ml. of the inoculum was injected intravenously into a sheep. The sodium hydroxide in a 6 per cent solution was added to an equal volume of virus-infected serum and thoroughly mixed. After being held at room temperature for five minutes, 8 ml. of the mixture was quickly diluted with buffered saline solution to a total volume of 30 ml., which was immediately injected intravenously into a sheep. The inactivation mixture of ethyl alcohol and virus-serum was prepared by adding 11.2 ml. of 95 per cent ethyl alcohol to 4 ml. of the virus-serum to obtain a 70 per cent ethyl alcohol concentration. This mixture was held at room temperature for five minutes, after which time the 15.2 ml. was injected intravenously into a sheep.

Controls.—To test the virulence of the virus-serum, 4 ml. was injected subcutaneously into a sheep. This animal was housed with the inoculated sheep. Two noninoculated sheep also were housed with the injected animals, and served as contact controls.

Challenge of Nonreactor Sheep.—The nonreactor sheep that had been inoculated with a virus-chemical mixture were held for 40 days postinoculation and then challenged with the homologous virus, New Mexico BT-52 strain.

Results

Effect of Chemical Agents on Embryo-Adapted Bluetongue Virus.—In the procedure followed in conducting the tests, agent W at a final concentration of 750 p.p.m., or greater, inactivated the bluetongue virus in each of the chicken embryo sources as shown (tables 1, 2, 3), but at a final concentration of 500 p.p.m. Agent W was effective in inactivating the virus only in undiluted chicken embryo supernatant fluid (table 2). In the one test using 3 per cent sodium hydroxide, this chemical inactivated the virus in undiluted embryo supernatant fluid (table 2). At the same concentration, it inhibited virus activity in the first five of seven tests where the virus was contained in undiluted ground chicken embryo (table 1).

Agent R at a final concentration of 5,000 p.p.m. inactivated the virus in undiluted chicken embryo supernatant fluid in the

TABLE 2—Effect of Exposure to Chemical Agents of Undiluted Chicken Embryo Supernatant Infected with Bluetongue Virus

Chemical agent	Final chemical concentration	Virus titer	Results of injection of eggs with virus chemical and chemical diluent previously incubated for 4 to 6 min. at 22 C.		Virus chemical subpassage; embryo deaths
			Virus chemical	Chemical diluent (controls)	
		Buffered saline diluent		Buffered saline solution	
Agent R	5,000 p.p.m.	$10^{6.9*}$	3/11†	2/20	0/9
Agent W	250 p.p.m.	$10^{4.3}$	22/22	2/21	.....
	500 p.p.m.	$10^{4.3}$	0/19	3/24	.....
	500 p.p.m.	$10^{4.9}$	3/16	1/15	0/7
	750 p.p.m.	$10^{4.9}$	2/11	3/19	0/12
Sodium hydroxide	2.0%	$10^{3.9}$	7/16	7/22	12/12
	3.0%	$10^{3.9}$	0/14	1/21	.....
Sodium carbonate	5.0%	$10^{3.9}$	19/19	0/19	.....
	10.0%	$10^{4.3}$	18/18	5/21	.....

* The virus titer is expressed as the chicken embryo l.d.$_{50}$ per 0.1 ml. † The numerator indicates the number of embryos dying from the second through the seventh day, and the denominator represents the number of eggs injected after discarding those containing embryos which died within the first 24 hours of injection.

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single test run (table 2). It was not found to be fully effective against the virus in chicken embryo supernatant fluid 10^{1} or in undiluted ground chicken embryo. Sodium carbonate at final concentrations of 5 and 10 per cent had no apparent effect on the virus contained in undiluted chicken embryo supernatant fluid. No appreciable differences were found when beef-heart infusion broth was used in place of buffered saline solution as the diluent for the prepared solutions of chemical agents and the virus materials (tables 1, 2, 3).

Effect of Chemical Agents on Bluetongue Virus in Sheep Serum.—The sheep used for determining the infectivity of virus-serum after an exposure to a final concentration of 750 p.p.m. of agent W, 3 per cent sodium hydroxide, or 70 per cent ethyl alcohol for 5 minutes at 22 C. did not show any febrile response or other clinical signs of bluetongue.

Discussion

The effectiveness of agents R and W, sodium hydroxide, and sodium carbonate at different concentrations as virucides for bluetongue virus in chicken embryo material was determined. In these trials, the reaction period of exposure for the virus-chemical was four to six minutes at 22 C. Longer exposure periods or even different temperatures during the exposure period of the virus-chemical mixtures might possibly have been factors that would have significantly affected the results of the trials.

The virus titers ranged from $10^{3.7}$ to $10^{4.9}$, with a range of $10^{4.0}$ to $10^{4.5}$ in the majority of the tests. This range of virus infectivity is considered satisfactory for chicken embryo-propagated bluetongue virus.

A number of subpassage tests were conducted to demonstrate the presence of the virus in inoculum prepared from embryos dying between the second and the seventh day postinoculation with the virus-chemical mixtures. The cause for the greater number of embryo deaths that occurred in a few of the chemical-diluent control tests was not determined. There were no embryo deaths in the eggs used for incubator controls in any of the tests.

It is apparent from the results obtained in these tests, with the exception of the two tests with sodium carbonate, that the chemicals at most of the concentrations used had some inactivating effect on the virus. It was of interest that the penetration of agent W (750 p.p.m.) was sufficient to

TABLE 3—Effect of Exposure to Chemical Agents of Chicken Embryo Supernatant 10^{1} Infected with Bluetongue Virus

Chemical agent	Final chemical concentration	Virus titer	Results of injection of eggs with virus chemical and chemical diluent previously incubated for 4 to 6 min. at 22 C.		Virus chemical subpassage; embryo deaths
			Virus chemical	Chemical diluent (controls)	
		B.H.I.B.* diluent		B.H.I.B.*	
Agent R	180 p.p.m.	$10^{4.0}$†	18/18**	3/23	.....
	2,500 p.p.m.	$10^{4.7}$	6/17	1/17	.....
	3,500 p.p.m.	$10^{3.8}$	2/19	0/19	12/12
	3,500 p.p.m.	$10^{4.7}$	3/17	2/17	11/11
	5,000 p.p.m.	$10^{4.5}$	1/19	0/20	.....
	5,000 p.p.m.	$10^{3.8}$	3/22	0/21	11/11
	5,000 p.p.m.	$10^{4.0}$	1/21	0/21	.....
Agent W	38.5 p.p.m.	$10^{4.0}$	20/20	2/23	.....
	750 p.p.m.	$10^{3.8}$	1/19	0/19	0/12
	750 p.p.m.	$10^{4.7}$	0/18	0/19	.....
	1,000 p.p.m.	$10^{4.5}$	0/21	1/22	.....
	1,000 p.p.m.	$10^{3.8}$	1/21	1/21	0/11
	1,000 p.p.m.	$10^{4.0}$	0/17	2/20	.....
Sodium hydroxide	2.0%	$10^{4.5}$	11/23	0/23	.....
	2.0%	$10^{4.7}$	0/15	1/15	.....
	2.0%	$10^{3.8}$	2/17	2/18	11/11
	2.0%	$10^{4.0}$	6/19	0/18	.....

* B.H.I.B. = Beef-heart infusion broth (Difco), pH 7.2. † The virus titer is expressed as the chicken embryo $l.d._{50}$ per 0.1 ml. ** The numerator indicates the number of embryos dying from the second through the seventh day, and the denominator represents the number of eggs injected after discarding those containing embryos which died within the first 24 hours of injection.

inactivate bluetongue virus suspended in undiluted ground chicken embryo where there was a considerable quantity of extraneous material as compared with that contained in embryo supernatant fluids.

The negative reaction of sheep to an intravenous injection of virus-chemical mixture indicated the virus had been inactivated by the chemical agent used. This determination was subsequently confirmed when each sheep reacted to challenge with the homologous virus, New Mexico BT-52 strain.

SUMMARY

The effectiveness of five chemicals (Rocal, agent R; Wescodyne, agent W; sodium hydroxide; sodium carbonate; and ethyl alcohol) against bluetongue virus was evaluated in tests involving the use of embryonating chicken eggs and sheep.

In each of the tests using embryonating eggs with four of the chemical agents in four- to six-minute inactivation periods, agent W at a final concentration of 750 p.p.m. or greater was the only one that completely inactivated the virus.

No difference in the results was noticed when beef-heart infusion broth (Difco), at a pH of 7.2, was used in place of buffered saline solution as the diluent.

In one test using sheep for the experimental animals, bluetongue virus in sheep serum was inactivated in a five-minute inactivation period by 750 parts per million of agent W, 3 per cent sodium hydroxide, or 70 per cent ethyl alcohol in final concentration.

References

[1] Alexander, R. A.: The Propagation of Blue-Tongue Virus in Developing Chick Embryo with Particular Reference to the Temperature of Incubation. Onderstepoort J. Vet. Sci. & Anim. Indust., *22*, (1947): 7–26.

[2] McKercher, D. G., McGowan, Blaine, Howarth, J. A., and Saito, Joseph K.: A Preliminary Report on the Isolation and Identification of the Bluetongue Virus from Sheep in California. J.A.V.M.A., *122*, (April, 1953): 300–301.

[3] Neitz, W. O.: Immunological Studies on Bluetongue in Sheep. Onderstepoort J. Vet. Sci. & Anim. Indust., *23*, (1948): 93–136.

[4] Reed, L. J., and Muench, H.: A Simple Method of Estimating Fifty Percent End Points. Am. J. Hyg., *27*, (1938): 493–497.

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Strain Variation in the Response of Sheep Nematodes to the Action of Phenothiazine. IV. Efficacy of Single Therapeutic Doses for the Removal of *Haemonchus Contortus*

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PREVIOUS INVESTIGATIONS by the authors [3, 4] demonstrated a significant level of resistance to small daily doses of phenothiazine in a strain of *Haemonchus contortus* that was isolated from a flock [11] with a worm control problem despite intensive use of the drug. This resistant strain B had a drug threshold four to eight times higher than the normal control strain A, which appeared to be similar to strains of *H. contortus* studied by other investigators.[7, 9, 13]

The present study on the efficacy of single therapeutic doses was undertaken to extend the evaluation of the drug resistance of strain B.

MATERIALS AND METHODS

Seven experiments were completed in which 42 helminth-free lambs (Cheviot ram crossed with grade western ewes) were used. In each experiment, 3 lambs per strain were infected, which provided 1 untreated control and 2 treated animals. The standard level of infection was 2,500 larvae from 7-day-old cultures of feces.

The present strains of *H. contortus* have been maintained in otherwise helminth-free lambs since the original isolation from the respective flocks in 1955.[4] In each experiment, the infections were established from the respective control animal of the preceding experiment except for experiments 1 and 5, in which the sources were donor lambs free of other helminth infections. The infections were maintained in the untreated donor lambs during the intervals between the yearly crops of lambs.

Dosage levels of phenothiazine were 0.1 and 0.25 Gm. per pound of body weight except in experiment 7, where the latter dosage was increased to 0.5 Gm./lb. The same lot of a commercial grade (N.F., green) of phenothiazine used throughout the study was the same as used in the previous investigation.[4] Particle size standards for this lot were: (1) 100 per cent must pass through a 16-mesh standard screen and (2) 85 per cent must pass through a 100-mesh screen. To insure administration, the individual doses were weighed, suspended in tap water, and administered via dose syringe equipped with a Whitlock nozzle.

Efficacy of treatment was evaluated by: (1) post-treatment decreases of 24-hour egg and larval counts in feces and (2) number of worms actually recovered at necropsy in relation to the number estimated to be present before treatment. The pretreatment estimates were based on the average egg production and sex ratio of the worms from the 7 untreated control animals for each strain.

Fecal collections were made every 24 hours from each animal from the third or fourth day before treatment until the seventh day after treatment. The techniques used for sampling the feces and making the egg and larval counts were the same as outlined earlier.[4] The animals were routinely slaughtered after the seven-day post-treatment observation period ended.

At necropsy, a careful search was made of the entire contents and lining of the gastrointestinal tract for nematodes. Haemonchus specimens were examined for type of vulval process in the female and length of spicules in the male.[16] In addition, gonadal squashes of males and females of both strains were prepared according to a modified method outlined by Bremner [2] for examination of the chromosomes. On the basis of the chromosomes, both strain A and strain B were typical *H. contortus*; however, details of these observations and the morphological features found in these strains will be reported separately.

RESULTS

The 24-hour egg and larval count data show that the three dosage levels were strikingly effective against strain A (table 1). The lowest dosage level (0.1 Gm./lb.) reduced egg counts to zero in 4 of 7 animals and by 96.8 to 99.8 per cent in the others. Larval counts were also reduced an average of 99.1 per cent in these animals.

For strain B, the 0.1-Gm./lb. dosage level was less effective in reducing the egg and larval counts (table 1). Changes in egg counts ranged from a 29.6 per cent increase in 1 lamb to a 100 per cent decrease in another. In the aggregate, the average

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decrease was 68.2 per cent. Larval counts in these lambs showed an average decline in the aggregate of 79.5 per cent after treatment.

In the aggregate, the greater reductions of the egg and larval counts for strain A over strain B were statistically significant beyond the 5 per cent level.

Dosages of 0.25 Gm./lb. were nearly 100 per cent effective in eliminating eggs and larvae from the feces after treatment of lambs infected with strain A (table 2). A high order of activity against strain B was also consistent for the 0.25-Gm./lb. dose level (table 2). Twenty-four hour egg and larval counts revealed reductions ranging from 92.2 to 100 per cent and 88.1 to 100 per cent, respectively. Statistical analysis revealed that these reductions for strain B were not significantly different from strain A.

In contrast, the 0.5-Gm./lb. dose was somewhat less effective in the lamb with strain B (711M, table 2). Here, the egg and larval counts were reduced 87.9 and 97.3 per cent, respectively, whereas complete suppression was achieved in the lamb infected with strain A.

Similar egg and larval count data in the 7 untreated control animals for each strain are also summarized (tables 1, 2). These averages showed little change for the periods of observation corresponding with the treated animals.

In the untreated control lambs, the preslaughter egg and larval counts divided by the number of female worms recovered at necropsy gave an estimate of the eggs and larvae per female worm per day for each strain. These averaged 6,600 and 2,870, respectively, for strain A and 5,360 and 2,070, respectively, for strain B. Statistical analysis showed no significant difference between these strain means. Likewise, there was no significant difference between the mean preslaughter and the mean pretreatment daily egg production and larval development within a strain or between strains.

The preslaughter 24-hour egg production and larval development for strain A female worms in lambs receiving the 0.1-Gm./lb. dosage averaged 1,670 and 800, respectively (table 1). These were significantly lower (5% level) than the values for the strain A controls. Worms were present in only 1 of the lambs infected with strain A after treatment at the 0.25-Gm./lb. level and the estimated daily egg production and larval development of 990

TABLE 1—Reduction of Fecal Egg and Larval Counts Following 0.1-Gm./lb. Dosages of Phenothiazine Given to Lambs Infected with Haemonchus Contortus

Exp. No.	Lamb No. & Sex	Total dose (Gm.)	24-hour egg and larval counts: Pretreatment* (thousands) Eggs	Pretreatment* (thousands) Larvae	Preslaughter† (thousands) Eggs	Preslaughter† (thousands) Larvae	Reduction (%) Eggs	Reduction (%) Larvae	No./female/24 hours preslaughter Eggs	No./female/24 hours preslaughter Larvae
STRAIN A (TREATED)										
1	69F	6.0	2,495	760	80	43	96.8	94.3	1,750	930
2	613F	5.8	1,587	857	0	0	100.0	100.0	.....	.....
3	624F	7.2	896	470	0	0	100.0	100.0	.....	.....
4	64M	7.9	580	433	0	0	100.0	100.0	.....	.....
5	716F	4.9	9,042	3,493	43	6	99.5	99.8	3,910	550
6	713M	5.5	2,686	1,783	0	3	100.0	99.8	.....	430
7	715F	4.5	3,494	2,463	7	9	99.8	99.6	1,000	1,290
	Average	6.0	2,969	1,466	19	9	99.4	99.1	1,670	800
	Untreated**	.....	2,144	1,100	2,257	960	+5.3‡	12.7	6,600	2,870
STRAIN B (TREATED)										
1	614M	5.5	827	179	115	17	86.1	90.5	1,200	180
2	639F	7.5	1,684	1,004	652	369	61.3	63.2	4,700	2,650
3	624F	7.7	4,336	1,729	740	471	82.9	73.1	6,320	4,030
4	622F	8.4	428	340	0	0	100.0	100.0	.....	.....
5	717M	5.6	5,320	4,756	6,894	2,159	+29.6‡	54.6	9,470	2,970
6	739M	6.0	1,061	390	20	11	98.1	97.2	4,880	2,750
7	712F	4.6	3,629	2,164	768	482	78.8	77.7	6,510	4,080
	Average	6.5	2,469	1,509	1,313	501	68.2	79.5	5,510	2,780
	Untreated**	.....	3,276	1,438	2,780	1,012	15.1	29.6	5,360	2,070

* Average of three or four counts during three to four day pretreatment interval; † Average of four counts during four to seven day post-treatment interval; ** Average of 7 lambs; ‡ Increase.

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TABLE 2—Reduction of Fecal Egg and Larval Counts Following 0.25-Gm./lb. Dosages of Phenothiazine Given to Lambs Infected with Haemonchus Contortus

Exp. No.	Lamb No.	Total dose (Gm.)	24-hour egg and larval counts: Pretreatment* (thousands) Eggs	Pretreatment* (thousands) Larvae	Preslaughter† (thousands) Eggs	Preslaughter† (thousands) Larvae	Reduction (%) Eggs	Reduction (%) Larvae	No./female/24 hours preslaughter Eggs	No./female/24 hours preslaughter Larvae
STRAIN A (TREATED)										
1	63F	14.9	1,812	375	0	0	100.0	100.0	.....	.....
2	623F	17.8	737	285	0	0	100.0	100.0	.....	.....
3	61M	18.8	194	101	0	0	100.0	100.0	.....	.....
4	66F	21.0	496	414	0	0	100.0	100.0	.....	.....
5	79F	13.6	5,924	2,506	2	0	99.9	100.0	990	150
6	710F	13.3	184	73	0	0	100.0	100.0	.....	.....
Average		16.6	1,558	626	0	0	100.0	100.0	990	150
7	73M	24.0**	551	536	0	0	100.0	100.0	.....	.....
Untreated‡		.....	2,144	1,100	2,257	960	+5.3	12.7	6,600	2,870
STRAIN B (TREATED)										
1	610F	17.0	1,909	540	148	64	92.2	88.1	4,920	2,130
2	623M	15.3	2,012	925	0	0	100.0	100.0	.....	.....
3	67M	19.3	1,488	864	65	49	95.6	94.3	2,940	2,230
4	612M	18.5	4,283	2,463	92	43	97.9	98.3	4,390	2,050
5	76F	14.3	3,651	1,704	0	0	100.0	100.0	.....	.....
6	710F	14.5	1,098	599	82	47	92.5	92.2	6,860	3,920
Average		16.5	2,407	1,183	65	34	96.4	95.5	4,780	2,580
7	711M	24.5**	2,879	2,220	349	59	87.9	97.3	2,730	460
Untreated†		.....	3,276	1,438	2,780	1,012	15.1	29.6	5,360	2,070

* Average of three or four counts during three to four day pretreatment interval; † Average of four counts during the four to seven day post-treatment interval; ** Dosage rate = 0.5 Gm./lb.; ‡ Average of 7 lambs.

and 150 per female worm (table 2) are considerably lower than those of the controls. In contrast, no significant differences were observed in the average daily egg production and larval development of the strain B females as a result of treatment with either the 0.1-Gm. or 0.25-Gm./lb. dosage levels (tables 1, 2).

In addition to the observations on the average daily egg production per female worm, the sex ratios of the worms from the untreated animals (tables 3, 4) were used to estimate the number of male and female *H. contortus* present in the animals before treatment. A comparison of these pretreatment estimates with the number of worms recovered at necropsy after treatment provided a second means of determining removal efficacy.

On this basis, the 0.1-Gm./lb. dosage level was also much more effective in removing strain A than strain B (table 3). Total doses of the drug were small and averaged 6.0 Gm. in strain A lambs and 6.5 Gm. in strain B lambs. Removal of strain A male and female *H. contortus* was of a consistently high order, ranging from 61.7 to 100 and 87.8 to 100 per cent, respectively. Corresponding values for strain B were of a much lower order and ranged from 25.3 to 98.7 and 26.7 to 100 per cent, respectively. In the aggregate, the differences between the means for the strains were not statistically significant.

The 0.25-Gm./lb. dosage level was nearly 100 per cent effective in removing strain A, as only 15 *H. contortus* worms were recovered from 1 of 6 animals at necropsy (table 4). Removal of strain B was less effective and more variable, ranging from 46.2 to 100 per cent and 91.6 to 100 per cent for the males and females, respectively. In the aggregate, the differences between the mean values for the strains were not statistically significant.

The 0.5-Gm./lb. dosage level was 100 per cent effective against strain A but showed a lack of effectiveness against strain B (table 4). Only 44.1 per cent of the males and 76.2 per cent of the females were removed from the single animal treated at this level.

Female *H. contortus* worms were consistently more effectively removed than the males. The sex ratios of the parasites from the untreated animals of both strains were consistently close to 1:1 (tables 3, 4). An average ratio (male/female) of 6.0:1.0 was

found in both strain A lambs treated at the 0.1-Gm./lb. and 0.25-Gm./lb. levels and strain B lambs treated at 0.25 Gm./lb. A ratio of 1.35:1.0 was recorded for the parasites from strain B lambs receiving the 0.1-Gm./lb. dosage. These deviations from the sex ratios observed in the untreated animals were statistically significant (x^2 test) beyond the 1 per cent level for both strains and the three dosage levels.

DISCUSSION

The lowest dosage level (0.1 Gm./lb.) of phenothiazine used in the present trials has been reported by several investigators [1, 8, 14, 15] to be sufficient for the highly effective removal of Haemonchus spp. from both sheep and cattle. In comparison with the other nematode parasites of sheep and cattle, Haemonchus spp. have generally been regarded [6, 10] as one of the most susceptible to phenothiazine. Strain A was effectively removed by the 0.1-Gm./lb. dose level and compares favorably with the foregoing reports, whereas strain B was only partially removed by 0.1-Gm./lb. doses and thus not only has a higher threshold for single doses of phenothiazine than strain A, but also the other strains of Haemonchus that have been studied.

The 0.25-Gm./lb. dosage level of phenothiazine has been generally employed for treating sheep in this country for the control of gastrointestinal nematodes.[6, 10] This level had a uniformly high efficacy of nearly 100 per cent against strain A, and averaged about 85 per cent against strain B. The latter is a higher order of activity than that generally observed in the field studies [11] on strain B, in which approximately the same dosage level of a different commercial preparation of the drug was used under field conditions. Even though this activity against strain B in the present tests might appear to be a satisfactory efficacy for practical therapeutics, much less effective control of strain B than strain A could reasonably be expected under field conditions. Further substantiation of this was evident in the relatively poor removal of strain B from an animal treated at twice the usual therapeutic level or 0.5 Gm./lb.

The consistently less effective removal of strain B than strain A by the single therapeutic doses is in accord with the previous comparison of these two strains [3, 4] to the low-level type of administration which indicated that strain B has a threshold 4 to 8 times higher than strain A. The range of dosage levels was more restricted in the present studies, but a comparable order of difference between the strains was evident. Strain A was effectively removed by 0.1

TABLE 3—Efficacy of 0.1-Gm./lb. Dosages of Phenothiazine Against Strain A and Strain B Haemonchus Contortus

Exp. No.	Lamb No.	Total dose (Gm.)	No. of worms: Pretreatment* Male	Pretreatment* Female	Necropsy Male	Necropsy Female	Removal efficacy (%) Male	Removal efficacy (%) Female
STRAIN A (TREATED)								
1	69F	6.0	384	378	147	46	61.7	87.8
2	613F	5.8	244	240	1	0	99.6	100.0
3	624F	7.2	138	136	0	0	100.0	100.0
4	64M	7.9	89	88	0	0	100.0	100.0
5	716F	4.9	1,392	1,370	156	11	88.8	99.2
6	713M	5.5	414	407	83	7	80.0	98.3
7	715F	4.5	538	529	37	7	93.1	98.7
	Average	6.0	457	450	61	10	89.0	97.7
	Untreated†	.....	330	325	349	344	.....	.....
STRAIN B (TREATED)								
1	614M	5.5	150	154	112	96	25.3	37.7
2	639F	7.5	305	314	150	139	50.8	55.7
3	624F	7.7	787	809	346	117	56.0	85.5
4	622F	8.4	78	80	1	0	98.7	100.0
5	717M	5.6	966	993	704	728	27.1	26.7
6	739M	6.0	193	198	50	4	74.1	98.0
7	712F	4.6	658	677	264	118	59.9	82.6
	Average	6.5	448	461	232	172	56.0	69.5
	Untreated*	.....	594	611	411	422	.....	.....

* Estimated from 24-hour egg counts. † Average of 7 lambs.

TABLE 4—Efficacy of 0.25 Gm./lb. Dosages of Phenothiazine Against Strain A and Strain B Haemonchus Contortus

Exp. No.	Lamb No.	Total dose (Gm.)	No. of worms				Removal efficacy (%)	
			Pretreatment*		Necropsy			
			Male	Female	Male	Female	Male	Female
STRAIN A (TREATED)								
1	63F	14.9	279	275	0	0	100.0	100.0
2	623F	17.8	114	112	0	0	100.0	100.0
3	61M	18.8	29	29	0	0	100.0	100.0
4	66F	21.0	76	75	0	0	100.0	100.0
5	79F	13.6	912	898	13	2	98.6	99.8
6	710F	13.3	28	28	0	0	100.0	100.0
	Average	16.6	240	236	2	0	99.2	99.9
7	73M	24.0†	84	85	0	0	100.0	100.0
	Untreated**	.....	330	325	349	344	.....	.....
STRAIN B (TREATED)								
1	610F	17.0	346	356	186	30	46.2	91.6
2	623M	15.3	365	375	0	0	100.0	100.0
3	67M	19.3	270	278	56	22	79.3	92.1
4	612M	18.5	777	799	158	21	79.7	97.4
5	76F	14.3	662	681	14	0	97.9	94.2
6	710F	14.5	199	205	90	12	54.8	94.2
	Average	16.5	437	449	84	14	76.3	95.9
7	711M	24.5†	522	537	292	128	44.1	76.2
	Untreated*	.....	594	611	411	422	.....	.....

* Estimated from 24-hour egg count. † Dosage rate = 0.5 Gm./lb. ** Average of 7 animals.

Gm./lb., whereas a similar efficacy against strain B was only achieved with 0.25 Gm./lb., and the action of 0.5 Gm. was incomplete in one trial. Thus, the thresholds of these two strains to single therapeutic doses of phenothiazine appear to differ at least 2.5 times.

Observations on the average 24-hour egg production and larval development per female on those worms surviving treatment were of additional interest in evaluating the relative differences between these strains. Both egg production and larval development of strain A were significantly lower during the four to seven day post-treatment interval than the untreated control values for strain A. In contrast, the egg production and larval development for strain B females surviving treatment were not significantly different from the strain B controls. This would also contribute to the less effective control of strain B compared with strain A under field conditions.

Reductions of total 24-hour egg and larval counts following treatment appeared to give a good evaluation of the removal action on the female worms in comparison with the worm recovery data. This was contingent upon the facts that (1) a very small percentage of strain A, in which the post-treatment egg production and larval development were significantly depressed, survived treatment; and (2) the post-treatment egg production and larval development of the relatively large numbers of surviving strain B female worms were not significantly depressed.

Post-treatment reductions of total 24-hour egg and larval counts tended to overestimate the removal action against male *H. contortus*. Relatively large numbers of males were recovered from some of the animals showing a marked reduction in egg and larval counts after treatment and were particularly evident in strain A lambs receiving the 0.1-Gm./lb. doses and in strain B lambs treated at the 0.25-Gm./lb. level. Thus, the present observations tend to associate the drug resistance with the ability of the females to survive, which was most apparent in strain B lambs treated at the 0.1-Gm./lb. level.

The action of phenothiazine in selectively removing females more effectively than males has been previously reported [3, 4, 13] for *H. contortus*. This phenomenon has also been noticed [12] following single doses of phenothiazine for *Trichostrongylus axei* infection in calves, while the converse has been recorded [5] for strongyles of horses fol-

lowing low-level phenothiazine treatment.

The relative differences between strain A and strain B that have been pointed out in the foregoing discussion were determined under test conditions which were as constant as was possible to arrange. Many factors such as age, sex, body weight, condition, nutritive state, and breeding of the experimental animals; dosage rate, method of administration, and physical properties of the drug; and age, size, and type of parasitic infection may play a role in determining a drug's efficacy. These were essentially the same for both strains in the present experimental design; however, these variables would exist under field conditions and may account for the variation between the more effective removal of strain B at the 0.25-Gm./lb. dosage level than was previously recorded [11] for this strain under field conditions at the same dose rate of phenothiazine.

The test infections of adult *H. contortus* that developed from the standard exposure of 2,500 larvae were quite variable, and worm burdens ranged from light to moderate. Efficacy in heavily infected animals, as are encountered in the field, may not necessarily be the same as observed in light to moderate infections. Comparative efficacies against these types of infection would be of interest and are planned for future investigation.

In the present trials, treatment was administered early in the course of the experimental infections, at a time when the animals were in good condition and the worm infections were fairly stable; *i.e.*, the "spontaneous" loss of worms was at a minimum. The latter was determined by the egg and larval counts on untreated animals in each experiment for the periods corresponding with the pre- and post-treatment intervals on the treated animals. On this basis, strain B showed a greater spontaneous loss than strain A, but no correction for these changes in the controls was used in the analysis of the egg and larval count data on the treated animals. Thus, in this comparison, the efficacy against strain B was somewhat overrated.

Calculating (from the preslaughter average egg production and sex ratio of the worms from the untreated control animals) the number of worms present before treatment tended to underestimate the number of worms in the strain A infected lambs because of the slight increase in average 24-hour egg counts between the pretreatment and preslaughter intervals. In contrast, the average 24-hour egg counts for the strain B controls decreased between the same intervals, which tended to give estimated pretreatment worm burdens higher (31%) than the number actually recovered at necropsy. No correction was made for this spontaneous loss of worms from the strain B lambs during the pretreatment and preslaughter interval; it was credited to the drug when calculating its efficacy against the worms. This analysis also overrated the activity of the drug against strain B.

The results of the present trials are in accord with the general body of evidence comparing these strains. The observations have been consistent both in direction and magnitude in showing the relative differences between response of these strains to phenothiazine, when either the low-level or single-dose type of administration was used. Until demonstrated otherwise, this relative degree of resistance of strain B must be regarded as exceptional and does not warrant excluding phenothiazine from sheep parasite control programs. However, it is noted that, the department's diagnostic section records, there was a marked increase during 1958 in the number of cases in which there was evidence of the lack of effectiveness of phenothiazine under field conditions. In general, the flock history for these animals indicates that phenothiazine-salt (1:9) was available and that several therapeutic doses were given at three to four week intervals, the last being within several days of the necropsy examination which revealed acute stomach worm infection to be the cause of death. Under similar circumstances, alternating drenches with "Cu-nic" mixture (copper sulfate-nicotine sulfate) and phenothiazine has provided a generally successful control program.

SUMMARY

The efficacy of single therapeutic doses of phenothiazine against a resistant strain B, and a control or nonresistant strain A, of *Haemonchus contortus* was studied in a series of seven experiments in which 42 helminth-free lambs were used.

Dosage at the rate of 0.1 Gm./lb. was highly effective against strain A, reducing post-treatment 24-hour egg and larval counts on feces 99.4 and 99.1 per cent, re-

spectively, and removing an average of 89.0 and 97.7 per cent of the male and female worms, respectively. Corresponding averages for this dosage level against strain B were 68.2 and 79.5 per cent reductions of the egg and larval counts, and 56.0 and 69.5 per cent removal of the male and female worms, respectively.

The 0.25-Gm./lb. dosage level was 100 per cent effective in reducing strain A egg and larval counts and removed an estimated 99.2 and 99.9 per cent of the male and female worms, respectively. This dosage was somewhat less effective against strain B, reducing the egg and larval counts an average of 96.4 and 95.5 per cent and removing 76.3 and 95.9 per cent of the male and female worms, respectively.

In single trials at the 0.5-Gm./lb. dosage level, complete removal of strain A worms was observed, whereas only 44.1 and 76.2 per cent of the strain B male and female worms were removed and egg and larval counts were lowered 87.9 and 97.3 per cent, respectively.

A significant reduction of average daily egg production and larval development of surviving females during the four to seven day post-treatment interval was observed for strain A but not strain B. Female worms of both strains and at all dosage levels were removed more effectively than the males.

The 2.5-fold difference between the thresholds of these two strains to single therapeutic doses in the present tests compares favorably with the four- to eight-fold difference to small daily doses used in previous studies. Since this relative resistance of strain B to phenothiazine is regarded as exceptional, other causative factors should not be overlooked in a flock with a parasite control problem.

References

[1] Anon.: Coordinated Trials with Phenothiazine Against Nematodes in Lambs. Imperial Agric. Bureau, Joint Publ. 4, (1943): 1–56.

[2] Bremner, K. C.: Cytological Studies on the Specified Distinctness of the Ovine and Bovine "Strains" of the Nematode *Haemonchus Contortus* (Rudolphi) Cobb (Nematoda: Trichostrongyloidae). Austral. J. Zool., *3*, (1955): 312–323.

[3] Drudge, J. H., Leland, S. E., Jr., and Wyant, Z. N.: Strain Variation in the Response of Sheep Nematodes to the Action of Phenothiazine. I. Studies on Mixed Infections in Experimental Lambs. Am. J. Vet. Res., *18*, (Jan., 1957): 133–141.

[4] Drudge, J. H., Leland, S. E., Jr., and Wyant, Z. N.: Strain Variation in the Response of Sheep Nematodes to Action of Phenothiazine. II. Studies on Pure Infections of *Haemonchus Contortus*. Am. J. Vet. Res., *18*, (April, 1957): 317–325.

[5] Drudge, J. H., Wyant, Z. N., and Elam, G. W.: Continuous Phenothiazine Therapy for Horses. II. A Taxonomic Study Following Four Years of Treatment. Am. J. Vet. Res., *16*, (Jan., 1955): 18–21.

[6] Edwards, J. T.: Phenothiazine 1942–1946—A Review and Bibliography. Imperial Bur. Agric., Joint Publ. 12, 1947.

[7] Gordon, H. McL.: A Note on the Possible Anthelmintic Value for Sheep of Phenothiazine Incorporated in Feed or Licks. J. Counc. Sci. Indust. Res. Austral., *15*, (1942): 54–55.

[8] Gordon, H. McL.: The Minimum Dose of Phenothiazine Required to Remove 100 per cent of *Haemonchus Contortus* from Sheep. Austral. Vet. J., *19*, (1943): 29–30.

[9] Haberman, R. T., and Shorb, D. A.: The Effect of Phenothiazine-Salt Mixtures on the Development of Parasite Larvae in the Feces of Sheep. N. Am. Vet., *23*, (1942): 318–321.

[10] Harwood, P. D. Parasitological Reviews. The Anthelmintic Properties of Phenothiazine. Exptl. Parasitol., *2*, (1953): 428–455.

[11] Leland, S. E., Jr. Drudge, J. H., Wyant, Z. N., and Elam, G. W.: Strain Variation in the Response of Sheep Nematodes to Action of Phenothiazine. III. Field Observations. Am. J. Vet. Res., *18*, (Oct., 1957): 851–860.

[12] Leland, S. E., Jr., Drudge, J. H., Wyant, Z. N., Elam, G. W., and Hutzler, L. B.: Studies on *Trichostrongylus Axei* (Cobbold, 1879). IV. Some Aspects of Treatment, Pathogenicity and Quantification of Experimental Infections of a Horse Strain in Calves. Am. J. Vet Res., *20*, (1959): In press.

[13] Page, K. W.: The Anthelmintic Effect of Phenothiazine Administered in Small Daily Doses to Lambs Having Heavy Infections of *Haemonchus Contortus*. J. Comp. Path. & Therap., *59*, (1949): 70–80.

[14] Porter, D. A.: Further Tests with Unconditioned Phenothiazine as an Anthelmintic in Cattle. Proc. Helminthol. Soc. Washington, *8*, (1941): 38–41.

[15] Riek, R. F.: The Use of Phenothiazine Against the Nematode Parasites of Cattle with Particular Reference to the Hookworm, *Bunostomum Phlebotomum*. Austral. Vet. J., *27*, (1951): 197–202.

[16] Roberts, F. H. S., Turner, H. N., and McKevett, M.: On the Specific Distinctness of the Ovine and Bovine "Strains" of *Haemonchus Contortus* (Rudolphi) Cobb (Nematoda: Trichostronglyoidae). Austral. J. Zool., *2*, (1954): 275–295.

XUM

Bacteremia in Pregnant Sheep Following Oral Administration of *Vibrio Fetus*

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VIBRIOSIS OF SHEEP, an acute infectious disease, is characterized by abortion during advanced stages of gestation, and is caused by *Vibrio fetus*. The disease is not evidenced by well-defined clinical signs preceding abortion. Following abortion and recovery from infection, abortions usually do not recur in the same animals.

Vibriosis may cause severe economic loss from abortions, which may exceed 70 per cent in infected flocks, from death of lambs, and from a mortality of approximately 5 per cent of infected ewes.

Although the pathogenesis of vibriosis in sheep is not thoroughly understood, transmission studies reveal that susceptible ewes in advanced stages of gestation are readily infected by oral inoculation with *V. fetus*-infected lamb tissues or cultures of *V. fetus* recently isolated from aborted lambs.

The objective of these studies was to determine the presence and duration of bacteremia in pregnant ewes following oral inoculation with *V. fetus*. Two experiments were conducted.

REVIEW OF LITERATURE

McFadyean and Stockman [6] reported producing abortion by inoculating ewes orally with infected lamb tissues or *V. fetus* cultures. They believed the oral route to be the most likely means of natural transmission. Stockman [8] reported later that abortions resulted when 2 healthy, pregnant ewes were permitted to share the same pen with infected ewes.

Jensen *et al.*[3] fed ovine fetal tissue infected with *V. fetus* to 23 ewes at the beginning of the fifth month of gestation and produced abortion in 17 ewes. No abortions resulted in an additional 68 ewes which were bred to rams originating from flocks of sheep which had histories of vibrionic abortions. The rams contained *V. fetus* in preputial secretions from natural exposure as well as from superimposed intrapreputial inoculation prior to the breeding season. Frank *et al.*[2] reported 100 per cent abortions in pregnant ewes following oral administration of *V. fetus*-infected tissues and fluids obtained from aborted lambs. Lee and Scrivner [4] induced four abortions in ewes by feeding various materials containing *V. fetus*. Lindenstruth *et al.*[5] produced three abortions in ewes from which *V. fetus* was recovered, by feeding hay and water inoculated with cultures of *V. fetus*. Miller and Jensen [7] isolated *V. fetus* from the blood of 1 of 2 rams that had been inoculated orally with *V. fetus*-infected viscera from aborted lambs.

MATERIALS AND METHODS

Experiment 1 (1956).—Ewes.—A total of 25 ewes, 2 and 3 years old, were randomly allotted to two lots, A and B. The 2-year-old ewes were purchased when they were yearlings and had been maintained in isolation approximately one year prior to use. The 3-year-old ewes each produced 1 normal lamb the previous year. Ewes from both sources originated from flocks which had no history of vibriosis. Cervical mucus samples were obtained from ewes of both age groups and examined bacteriologically for the presence of *V. fetus* prior to the breeding season. The ewes were bred to a healthy ram. Ewes of lot A were not inoculated. Ewes of lot B were each inoculated orally with approximately 30 Gm. of freshly ground tissues from aborted lambs infected with *V. fetus*. Inoculations were made at the beginning of the fifth month of gestation.

Ram.—The ram used for breeding originated from a flock having no history of vibriosis. Three preputial mucus samples from the ram were examined bacteriologically for *V. fetus* prior to the breeding season.

Blood Cultures.—Jugular blood samples (5 ml.) were collected and cultured. Two preinoculation and six postinoculation samples were studied. Blood was cultured in thiol medium, incubated under partial vacuum at 37 C. for 72 hours in an atmosphere containing 10 per cent nitrogen gas. Blood cultures were gram-stained and examined for *V. fetus*. Isolates were obtained on blood agar plates from positive blood cultures. Isolates were examined for H_2S and catalase activity.

Serum Samples.—Ten milliliter serum samples from 7 inoculated and 6 control ewes were sent to a diagnostic laboratory in Scotland and were tested for antibodies of ovine virus abortion by the complement fixation test.

Experiment 2 (1958).—Ewes.—A total of 49

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virgin 2-year-old ewes originated from a flock which had no history of infection with *V. fetus*. The animals were arranged in one lot and maintained in isolation for approximately six months prior to breeding. During the fifth month of gestation, they were inoculated orally with viable cultures of *V. fetus* from aborted lambs.

TABLE 1—Vibrio Fetus Bacteremia in Pregnant Sheep Following Oral Administration of Tissues from Aborted Lambs

Lots	No. of Sheep	Oral inoculation	Blood samples—Bacteremia			
			Preinoculation		Postinoculation	
			Pos.	Neg.	Pos.	Neg.
A	12	None	0	12	0	12
B	13	Infected tissue	0	13	6	7

Ram.—The ewes were bred to a ram originating from a flock of sheep having no incidence of vibriosis.

Inoculum.—Cultures used for inoculation of ewes were isolated from recently aborted lambs obtained from a single source. The inoculum consisted of 10 ml. of pooled, viable, cysteine-trypticase broth cultures of *V. fetus* adjusted photoelectrically to correspond to McFarland nephelometer tube 3.

Blood Cultures.—Prior to oral inoculation, two 5-ml. jugular blood samples from each ewe were cultured for *V. fetus*. Postinoculation blood samples were obtained from each ewe over a period of 46 days. Blood was cultured in cysteine trypticase broth containing 0.1 per cent agar. Cultures were incubated aerobically at 37 C. for 48 hours. Stained smear preparations were examined microscopically for *V. fetus*.

Cultures positive for *V. fetus* were subcultured on blood agar plates under partial vacuum in an atmosphere containing approximately 10.0 per cent nitrogen. Isolated colonies on blood agar plates were inoculated into screw-capped culture tubes of cysteine trypticase broth containing 0.1 per cent agar and incubated aerobically at 37 C. for 48 hours.

The cultures were determined to be *V. fetus* by biochemical tests,[1] with the exception of growing the microorganisms in cysteine trypticase medium without the aid of various gases or reduced oxygen tension.

RESULTS

Experiment 1.—Cervical and preputial samples were negative for *V. fetus*. Two preinoculation blood samples cultured from pregnant ewes of lots A and B were also negative for *V. fetus* (table 1).

Bacteremia occurred in 6 of 13 ewes inoculated with lamb tissues infected with *V. fetus*. In addition, *V. fetus* was isolated from the liver of 1 of the 6 ewes. Postinoculation blood samples from ewes of lot A were negative throughout the experiment. Serum samples were negative for enzootic ovine virus abortion.

Experiment 2.—Preinoculation jugular blood samples cultured from 49 pregnant ewes were negative for *V. fetus* (table 2).

Bacteremia was demonstrated in 32 of 49 pregnant ewes inoculated orally with cultures of *V. fetus*. The greatest incidence of bacteremia occurred during the third day following inoculation. A bacteremia was not demonstrated in 2 ewes until the twelfth and fourteenth days. *Vibrio fetus* was not found in any blood sample obtained after the fourteenth day following inoculation.

TABLE 2—Vibrio Fetus Bacteremia in Pregnant Sheep Following Oral Administration of Vibrio Fetus Cultures

Bacteremia	Blood samples																	
	Preinoculation days		Postinoculation days															
	2	0*	3	5	7	10	12	14	17	21	25	28	32	35	38	41	46	
No. positive	0	0	25	11	4	0	1	1	0	0	0	0	0	0	0	0	0	
No. negative	49	49	24	38	45	49	48	48	49	49	49	49	49	49	49	49	49	

* Blood samples obtained prior to inoculation.

DISCUSSION

Although the pathogenesis of vibriosis in sheep has not been fully determined, transmission studies have shown oral inoculation of *V. fetus* during advanced stages of gestation to be an effective method of transmission of vibriosis to susceptible ewes.

The presence of a bacteremia following the feeding of *V. fetus* confirms the assumption that one portal of entry for *V. fetus* into the animal's body is through the digestive tract.

The failure to demonstrate a bacteremia in all inoculated animals may be due to the time of blood collection, individual resistance to infection, contamination of blood by other organisms, or culture requirements.

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SUMMARY

In ewes inoculated orally at the beginning of the fifth month of gestation, bacteremia resulted in 6 (46.1%) of 13 ewes given tissues from aborted lambs infected with *Vibrio fetus* and in 32 (65.3%) of 49 ewes given *V. fetus* culture.

References

[1] Bryner, J. H., and Frank, A. H.: A Preliminary Report on the Identification of *Vibrio Fetus*. Am. J. Vet. Res., *16*, (Jan., 1955): 76–78.

[2] Frank, F. W., Bailey, J. W., Heithecker, D.: Experimental Oral Transmission of Vibrionic Abortion of Sheep. J.A.V.M.A., *131*, (Nov. 15, 1957): 472–473.

[3] Jensen, R., Miller, V. A., Hammarlund, M. A., and Graham, W. R.: Vibrionic Abortion in Sheep. I. Transmission and Immunity. Am. J. Vet. Res., *18*, (April 1957): 326–329.

[4] Lee, A. M., and Scrivner, L. H.: Experimental Work Upon Recent Outbreaks of Abortion in Ewes. Am. J. Vet. Res., *2*, (1941): 50–54.

[5] Lindenstruth, R. W., Ashcraft, J. B., and Ward, B. Q.: Studies on Vibrionic Abortion of Sheep. J.A.V.M.A., *114*, (April, 1949): 204–205.

[6] McFadyean, J., and Stockman, S.: Abortion in Sheep. In "Report of the Departmental Committee Appointed to Inquire into Epizootic Abortion." Part 3, Great Britain Board of Agriculture & Fisheries. His Majesty's Stationery Office, London, 1913.

[7] Miller, V. A., and Jensen, R.: Unpublished data, Colorado State University, Fort Collins, 1955.

[8] Stockman, S.: Vibrionic Abortion. J.A.V.M.A., *55*, (Aug., 1919): 499–504.

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Gross Cardiac Changes in Cattle with High Mountain (Brisket) Disease and in Experimental Cattle Maintained at High Altitudes

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HIGH MOUNTAIN or brisket disease is a syndrome in cattle residing at high altitudes of the western United States. The disease manifests itself as congestive failure of the right side of the heart and all signs and lesions are attributed to this pathological phenomenon. Since congestive failure of the right side of the heart also occurs at low altitude, where the pathogenesis is usually evident, the term "high mountain disease" more clearly designates the syndrome as it exists in cattle residing at high altitudes.

The incidence of this disease is estimated to be 0.5 to 1.0 per cent of animals ranging above 7,000 feet. The incidence in any one herd varies from year to year and, in some herds, may reach as high as 5 to 10 per cent. The disease occurs primarily during the fall, winter, and spring months and affects all age and sex groups, with a higher incidence in calves less an 1 year old.

The disease, usually developing slowly, presents signs characteristic of congestive failure of the right side of the heart. The animal may first be depressed and isolated from the herd. As the disease progresses, subcutaneous edema develops, starting in the ventral pectoral region and extending posteriorly along the ventral abdominal wall and anteriorly to the intermandibular space. Pronounced ascites and marked distention and pulsation of the jugular vein are usually present. A dark fluid diarrhea often develops as the condition progresses. Respiration is labored and increased in rate, and the animals may appear cyanotic. They are reluctant to move, may become recumbent and, upon forced exertion, may collapse and die. The disease may progress rapidly to fatal termination within a week, or it may take a protracted course extending up to 12 weeks. Some animals recover spontaneously when moved to a lower altitude.

Gross lesions of animals suffering with high mountain disease relate to congestive failure of the right side of the heart. The most striking picture is that of generalized anasarca with infiltration of transudate into the subcutaneous tissues, skeletal musculature, and body cavities. Usually, extensive hydrothorax and ascites are seen.

The liver lesions are those of chronic passive congestion and range from an early "nutmeg" appearance to severe central or so-called "cardiac" cirrhosis. Marked edema of the mesentery and alimentary tract wall is often present. The latter change accounts for the profuse fluid diarrhea that frequently accompanies this disease.

The lungs show extensive areas of atelectasis and, in some cases, limited emphysema. Atelectasis is usually more pronounced in the dependent portions of the lungs and is probably secondary to the hydrothorax. Pneumonia is sometimes present. In animals less than 1 year old, thrombi are occasionally found in the pulmonary arteries.

The heart shows marked right ventricular hypertrophy and dilatation. The anatomical cardiac apex, as normally formed by the left ventricle, is frequently displaced to the left, giving the heart a rounded contour. The wall of the right ventricle may be greatly thickened or moderately thin, depending on the extent of the dilatation that has occurred. The moderator band of the right ventricle is often hypertrophic and may measure ¾ of an inch in diameter.

As this disease appears to manifest itself as congestive failure of the right heart, the present studies were undertaken to determine the location and degree of gross cardiac changes associated with clinical cases of high mountain disease and in experimental cattle residing at high altitudes.

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These data were submitted in part to the Graduate School by the senior author in partial fulfillment of requirements for the M.S. degree.

REVIEW OF LITERATURE

Glover and Newsom first described brisket or high mountain disease of cattle in 1915.[6,7,18] They stated that the malady had been recognized in the mountainous regions of Colorado since 1889. The syndrome they described presented the typical history, signs, and necropsy lesions as seen today. Hearts from animals with clinical cases were enlarged, dilated, and usually flaccid. They also observed that the hearts of normal cattle originating from high elevations were heavier and thicker than those of animals originating from lower altitudes.

Caparó *et al.*[3] described the condition known as chronic mountain sickness of cattle as it exists in Peru at elevations of 11,000 to 13,000 ft. In that country, the disease has been known for many years. The environmental conditions, signs, and lesions are similar to those described by Glover and Newsom. The cardiac changes observed were hypertrophy and dilatation with a more pronounced involvement of the right side of the heart.

In 1950, Caparó [2] described mountain sickness in sheep in Peru at altitudes of approximately 13,000 ft. The condition, similar to high mountain disease of cattle, presented signs and lesions of congestive failure of the right side of the heart. At necropsy, the hearts of affected animals showed right ventricular and atrial dilatation. The hearts from 3 apparently normal animals at the high altitude were heavier than those from normal lambs at sea level.

Pierson and Jensen [15] stated that high mountain disease of cattle is essentially a congestive failure of the right side of the heart and is characterized by hypertrophy and dilatation of the right side of the heart. Ryff [15] reported this change in only 20 of 47 cases.

Sera and Jensen [19] measured histologically the transverse diameters of cardiac muscle fibers of hearts from normal cattle and from those with high mountain disease. Their work indicated that the muscle fibers of the right ventricle were markedly hypertrophic in the diseased heart, while those of the left ventricle showed no consistent alteration from normal.

In 1918, Glover and Newsom [8] compared the heart weights of 224 normal cattle raised at high altitudes with those of 138 animals raised at approximately sea level. Converted to a 1,000-lb. carcass weight basis, the hearts of animals raised at high altitudes averaged 0.897 lb. heavier than those from the low altitudes. They also compared the heart weights from 1 normal and 1 diseased animal. Both animals were 4 months old and approximately the same weight. The weight of the heart of the normal animal was 1.25 lb., while that of the animal with high mountain disease was 3.25 lb.

Van Liere [23] studied the effect of prolonged hypoxia on the heart weight in guinea pigs. Fifteen adult animals were kept for 20 to 105 days at oxygen tensions simulating those found at an altitude of 14,000 to 18,000 ft. The heart weights were compared with those of 73 animals kept under normal conditions. The heart-body weight ratio was increased an average of 155 per cent in animals maintained under the hypoxic conditions. Microscopic study of the hearts showed cardiac muscular hypertrophy in 3 of 4 hypoxic animals. The heart of the fourth animal showed "atrophy preceded by hypertrophy." The author postulated that the cardiac hypertrophy might be accounted for by either or both of two causes: (1) overwork due to increased blood viscosity and heart rate; (2) injury to the muscle fibers due to hypoxia.

Tappan and Reynafarje [21] compared the heart weights of 59 guinea pigs native to 14,900-ft. elevation with those of 83 guinea pigs native to sea level. The cardiac weights were transposed into ratios by the following formula: heart weight/body weight x 100. The hearts of those from high altitudes were heavier ($P<0.001$) than those from sea level. They also translocated 21 sea-level animals to the altitude of 14,900 ft. and kept them there for 0 to 123 days, with an average of 45 days. The hearts of these animals were likewise heavier ($P<0.001$) than those of controls at sea level.

Stickney and Van Liere,[20] in referring to Rotta's work,[17] stated that the heart weights of guinea pigs varied directly with altitude above 12,200 ft. and were 30 per cent greater at 14,900 ft. than at sea level. A diminished number of cardiac capillaries with an increased diameter of the muscle fibers was seen histologically.

Atland and Highman [1] exposed rats intermittently for four-hour intervals to a simulated altitude of 25,000 ft. There were 10 animals per exposure group. After nine months, the heart weights for those exposed daily were 62 per cent above normal; those exposed every two days were 20 per cent above normal; and those exposed every three days were 13 per cent above normal. The heart weights of those groups exposed at 5-, 7-, and 10-day intervals did not differ significantly from normal.

Van Liere and Stickney [22] exposed dogs intermittently to simulated altitudes of 12,000 to 18,000 ft. They found that there was no significant cardiac hypertrophy, as the heart weights of the experimental animals, expressed in grams per kilogram body weight, did not vary from the controls.

Moore and Price [12] exposed rats to elevations of 600 and 7,500 ft. for three months and to an elevation of 14,600 ft. for two months. No alteration in heart-body weight ratio was noticed.

Kerwin [10] radiographically observed the hearts of 273 normal Peruvian males residing at high altitude and compared them with hearts of normal white inhabitants residing at lower altitudes. The cardiac silhouette was found to be larger in the high-altitude group.

Rotta [17] made radiographic and electrocardiographic observations of the hearts of human beings living at an elevation of approximately 14,700 ft. Radiographic determinations on 400 individuals revealed that 69.5 per cent of the hearts had an

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increased transverse diameter and that 67.0 per cent had an increased frontal diameter. Also, 65 per cent of those with enlarged hearts had right heart prominence. Electrocardiographic measurements on 100 individuals revealed that the QRS complex had deviated to the right in 59 per cent and to the left in 21 per cent. From these data, the author concluded there was a tendency toward hypertrophy of the right side due to overwork of the heart, secondary to pulmonary alterations.

Penaloza [14] studied the electrocardiographic changes occurring in ten human subjects who had originated from sea level and who had resided at an altitude of 14,000 ft. for one year. The alterations were characteristic of right ventricular hypertrophy. The author also referred to some of his unpublished work, which indicated that the native residents at that altitude have an even greater degree of right ventricular hypertrophy.

Graybiel *et al.*[9] observed cardiac changes in four men who were partially acclimatized over a period of a month to a simulated altitude of 22,500 ft. The heart size, as determined by teleroentgenography, diminished slightly, and it was concluded that during early stages of high altitude acclimatization, hypertrophy and dilatation of the heart do not occur.

De Castro and Iglesias [4] studied, at necropsy, the hearts from 30 human beings who were acclimatized to an altitude of approximately 13,000 ft. Eight showed right ventricular hypertrophy, one showed left ventricular hypertrophy, and eight showed a tendency toward right ventricular hypertrophy. They concluded that: "Permanent residence at high altitudes is characterized in an appreciable number of cases, almost a majority, by a discrete hypertrophy of the right ventricle of the heart."

Reissmann [16] measured the blood volumes of 8 dogs that were acclimatized for ten to 12 weeks in decompression chambers to an altitude of 20,000 ft. The plasma level decreased slowly to a level of 75 per cent of the normal value, and the cell volume increased to 170 per cent of the normal value. The total blood volume increased to a level of 125 per cent of the normal value.

Changes in pulmonary arterial pressures of cats under the stimulus of hypoxia were observed by von Euler and Liljestrand.[5] They found that when anesthetized animals were forced to breath 10 to 11 per cent oxygen in nitrogen, an invariable rise in the pulmonary arterial pressure occurred. Lewis and Gorlin [11] observed a similar phenomenon in dogs breathing hypoxic mixtures.

MATERIALS AND METHODS

The 119 cattle, from which the hearts for this study were obtained, were of three general groups: 41 had clinical cases of high mountain disease; 39 were experimental cattle residing at high altitudes; and 39 were experimental cattle residing at low altitudes.

TABLE 1—Ventricular Ratios in Cattle with High Mountain Disease Versus Normal Cattle

No. of cattle	Value	RV/T	LV/T	S/T
NORMAL				
39	Mean	0.251	0.480	0.269
	Range	0.210–0.284	0.429–0.500	0.240–0.321
	Standard deviation	0.011	0.021	0.020
HIGH MOUNTAIN DISEASE				
41	Mean	0.435	0.317	0.248
	Range	0.308–0.554	0.228–0.446	0.213–0.285
	Standard deviation	0.087	0.046	0.019
	"t" value*	13.087	20.064	4.949
	Probability	<0.001	<0.001	<0.001

* Value of "t" for difference between means.

Cardiac Measurements.—The following procedure was employed in the processing of all hearts. The organ was first examined for changes such as pericarditis, myocarditis, valvular defects, and developmental abnormalities that might be involved in the pathogenesis of cardiac hypertrophy. If such alterations were present, the heart was discarded from this study.

The organ was then divided into three components: right ventricle, left ventricle, and septum. The atria, valvular elements, and coronary fat were trimmed from each portion, leaving an isolated mass of ventricular muscle. Each component was weighed, and ratios were obtained by dividing the weight of the portion of interest by the total ventricular weight.

To facilitate the location of the cardiac changes the following ratios were used:

1) RV/T = ratio of weight of right ventricle divided by total ventricular weight.

2) LV/T = ratio of weight of left ventricle divided by total ventricular weight.

3) S/T = ratio of weight of septum divided by total ventricular weight.

Study of Clinical Cases.—The hearts from 41 cattle with high mountain disease were processed in the manner described previously. All of these animals originated from high altitudes and presented typical histories, signs, and lesions of high mountain disease. Fresh material was available

from 27 such animals, while formalin-preserved specimens constituted the remaining 14.

The data obtained from the animals with clinical cases were compared with those from 39 normal animals which resided at low altitude. The latter group comprised the low-altitude lots in the experiments of 1956 and 1957.

Experimental Studies.—During each of the years 1956 (experiment 1) and 1957 (experiment 2), a 2 x 2 factorial experiment was designed to study the effect on cattle of: (1) residence at high altitude and (2) showers of autogenous, fibrinous emboli into the pulmonary vascular bed. The first factor was chosen because of the association of high mountain disease with residence at high altitude. The second treatment, fibrinous emboli, was used because of the occasional thrombosis of the pulmonary arteries in young animals affeced with the disease.

In each experiment, 40 cattle were used. The animals were yearling Hereford steers of uniform breeding, weighing 500 to 600 lb., originating from altitudes of 5,000 ft. or less. They were randomly allotted by weight and source into four treatment lots of 10 animals each. The following diagram depicts the experimental design for both trials.

		Altitude, Treatment A	
		0	1
Emboli, Treatment B	0	(1) Low altitude No emboli	a High altitude No emboli
	1	b Low altitude Emboli	ab High altitude Emboli

The high-altitude treatment was accomplished by pasturing the animals at an elevation of approximately 9,000 ft. The animals designated to remain at the low altitude were pastured at a 5,000-ft. elevation. In both experiments, animals were pastured at these elevations for approximately four months.

The fibrinous emboli for the second treatment were prepared from the blood of each recipient animal. The blood was drawn aseptically by jugular venipuncture and allowed to clot. Twenty four hours later, the contracted clot and serum were placed in a Waring blendor and ground for approximately five seconds. The resulting clots, suspended in serum, were of such a size that they would pass through a 14-gauge, but not a 16-gauge needle. This suspension was then reinjected into the jugular vein of the donor animal.

In experiment 1, one administration of the suspended fibrinous emboli prepared from 100 ml. of blood was given before the animals were placed at their respective elevations. In experiment 2, the emboli treatment was administered four times throughout the experiment at approximately three-week intervals. The first administration was given after the animals were placed at their respective altitudes. The dosage in the latter experiment was the suspended fibrinous emboli prepared from blood drawn at the rate of 0.2 ml. per pound of initial body weight.

At the termination of each experiment, the animals were slaughtered at commercial establishments and the hearts were collected for measurement.

Analysis of Data.—The results obtained from the comparison of the hearts of diseased animals with those of normal animals were analyzed by Student's "t" test. The data obtained from the factorial experiments were subjected to analysis of variance with a subdivision into orthogonal comparisons.

RESULTS

Study of Clinical Cases.—The results from the comparison of heart ratios obtained from animals with clinical cases of high mountain disease with those obtained from normal animals are given (table 1).

Compared to the normal heart (fig. 1), all hearts from cattle with clinical cases

Fig. 1—Normal bovine heart—the right ventricle is to the right of photograph.

showed evidence of gross right ventricular hypertrophy or dilatation or both (fig. 2–4). In the majority of hearts, this process was developed to the extent that the normal cardiac apex, as formed by the tip of the left ventricle, was shifted to the left. When this change occurred, the apex of the heart

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appeared either bifid or, in extreme cases, was formed by the right ventricle.

A histogram of the dispersion of RV/T ratios in normal hearts and those affected by high mountain disease is presented (fig. 5).

Experimental Studies.—At the termination of both experiments, all animals appeared normal. No gross cardiac changes could be detected by visual examination.

Experiment 1.—The statistical analysis of pertinent data obtained from experiment 1 is presented (table 2).

TABLE 2—Experiment 1, Analysis of Variance (1956)

Measurement	Key out	Effect	df	Probability
Initial body weight (lb.)	Mean	509.0		
	A	−9.0	1	
	B	−5.0	1	
	AB	+19.0	1	
	Error	.....	36	
Final body weight (lb.)	Mean	616.0		
	A	−41.0	1	
	B	11.0	1	
	AB	−37.0	1	
	Error	.....	35*	
RV/T	Mean	0.256		
	A	0.049	1	<0.001
	B	−0.001	1	
	AB	−0.009	1	
	Error	.....	34†	
LV/T	Mean	0.476		
	A	−0.055	1	<0.01
	B	0.027	1	
	AB	0.031	1	
	Error	.....	34†	
S/T	Mean	0.268		
	A	0.007	1	
	B	−0.027	1	
	AB	−0.019	1	
	Error	.....	34†	
Right ventricular weight (Gm.)	Mean	201.0		
	A	45.0	1	<0.01
	B	15.0	1	
	AB	−17.0	1	
	Error	.....	34†	
Left ventricular weight (Gm.)	Mean	376.0		
	A	−23.0	1	
	B	41.0	1	
	AB	−7.0	1	
	Error	.....	34†	
Septum weight (Gm.)	Mean	210.0		
	A	13.0	1	
	B	−3.0	1	
	AB	−29.0	1	<0.05
	Error	.....	34†	

A = effect of altitude; B = effect of emboli; AB = effect of interaction of altitude and emboli.
* Loss, one degree of freedom; † loss, two degrees of freedom.

One animal from treatment group B (low altitude-emboli) died from septic pulmonary embolism and thrombosis during the course of the experiment. The mean from the measurements obtained from the other 9 animals in this group was substituted for these lost data, and a reduction of one degree of freedom in the error term was made.

Fig. 2—Heart from a cow with high mountain disease. The right ventricle is to the right of photograph.

One of the hearts from an animal in treatment group AB (high altitude-emboli) was destroyed at the time of slaughter without obtaining accurate ventricular ratios. Again, the group mean was substituted for the missing data and a reduction of an additional degree of freedom in error was made.

There was no significant difference in ventricular ratios due to the effect of the emboli treatment or its interaction with altitude treatment. The actual septal weight, however, decreased due to the effect of the interaction of altitude and emboli. This decrease was barely significant at the 0.05 level. The standard deviation of the RV/T ratio in the 39 normal hearts (table 1) is about half that of the standard deviation of the LV/T and S/T ratios. This variation is present because the dissecting technique of separating the right ventricle from the heart is more easily and accurately accomplished than that of separating the left ventricle and septum. Thus, it is felt that the low significance of the decrease in septal weight due to the altitude-emboli interaction is one of method rather than one of treatment.

With these assumptions, and since there was no significant difference in ventricular ratios due to the main effect of emboli, it is statistically valid to observe the results

considering only one treatment—altitude—at two levels. This comparison is shown (table 3).

Experiment 2.—The statistical analysis of data obtained from experiment 2 is presented (table 4). Again there was no statistical difference due to the emboli treatment and, therefore, the results are grouped (table 5) according to the two levels of altitude.

DISCUSSION

The review of literature indicates that the predominant visual cardiac changes in high mountain disease of cattle are those of hypertrophy and dilatation of the right ventricle. These changes are in keeping with the generalized signs and lesions of the disease.

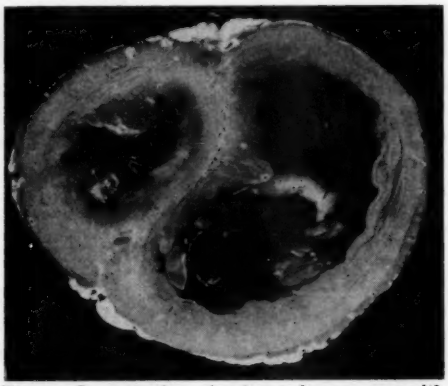

Fig. 3—Cross section of a heart from a cow with high mountain disease. Observation is toward the apex of the ventricles. The wall and moderator band of right ventricle are hypertrophied. The right ventricle is to the right of the photograph.

The literature also indicates that some degree of cardiac hypertrophy may occur when animals reside for a considerable time in rarefied atmospheres similar to those found at the higher elevations. More recent work in the field of human medicine has shown that, in people native to the higher elevations, there is a tendency for this hypertrophy to be located in the right ventricle.

Total heart weight varies directly with the body weight of an animal. Therefore, measuring and locating gross changes within ventricular muscle indicative of hypertrophy necessitates a method whereby the actual ventricular weight is not the unit of measurement. This may be accomplished by obtaining the ratio between the ventricular component of interest and the total ventricular weight.

Clinical Cases.—The highly significant increase in the RV/T ratio in the hearts from cattle with clinical cases of high mountain disease is indicative of marked right ventricular hypertrophy (table 1). The fact that there was a correspondingly significant decrease in the LV/T ratio illustrates a serious statistical problem which is encountered when dealing with ratios; namely, it is difficult to know whether the numerator or the denominator of the ratio is the factor which is changing. This answer could not be obtained from the comparison of the actual weight of the ventricular components, because the animals varied greatly in size and age. No grossly visible changes in the left ventricles of the hearts from cattle with clinical cases could be discerned, while the right ventricular alterations from normal were striking. It was therefore concluded that the increase in the RV/T ratio in the animals with clinical cases was due to a severe right ventricular hypertrophy. This evaluation is supported by the observations of Sera and Jensen.[19]

The histogram (graph 1) indicates that not only is the RV/T ratio increased in all cattle with clinical cases, but the distribution curve of this ratio is markedly flattened, ranging from mild to advanced hypertrophy. The distribution curve of the control or normal animals, however, shows

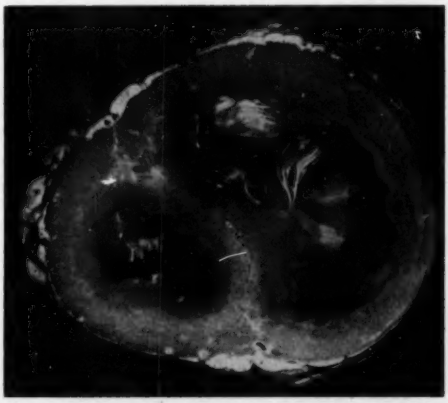

Fig. 4—Cross section of same heart shown in Fig. 3. Observation is toward the A-V valves. The right ventricle is to the right of the photograph.

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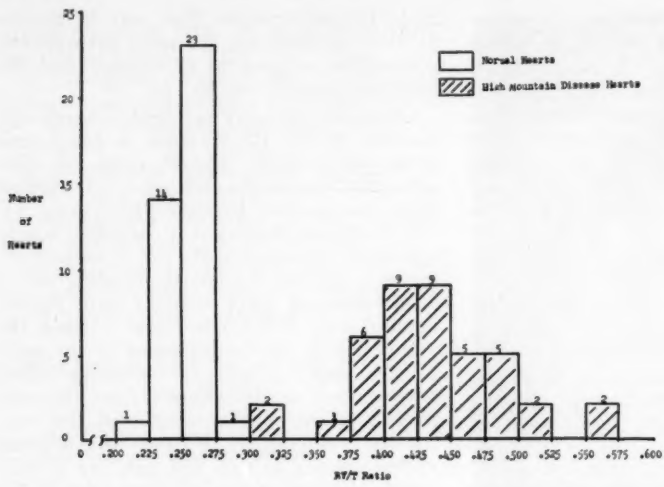

Graph 1—The RV/T ratios of hearts of a normal cattle and those with high mountain disease.

distinct kurtosis, with 37 of the 39 ratios falling in either of two adjacent class limits. The lack of kurtosis of the curve observed for the diseased hearts is in keeping with the variation in the severity of the syndrome as it occurs in a normal population.

The highly significant decrease in the S/T ratio of the hearts from animals with clinical cases may indicate a mild degree of hypertrophy of the septal component. The mean increase in the RV/T ratio was 42 per cent.

Because the data is in the form of ratios, the summation of the percentage decrease in the LV/T and S/T ratio must likewise equal 42 per cent—a minus 34 per cent for the LV/T ratio and a minus 8 per cent for the S/T ratio. If there had been no alteration in the actual weight of the left ventricle and septum, these ratios would have maintained the same proportion to one another. Had this been the case, the LV/T ratio would have decreased 27 per cent and the S/T ratio would have decreased 15 per cent.

In these data, the LV/T ratio decreased 34 per cent. If the assumption is made that there was no change in the actual left ventricular weight, the decrease of 8 per cent by the S/T ratio actually indicates a mild degree of hypertrophy in this component.

This change is both anatomically and physiologically logical. The septum forms the medial wall of both the ventricular chambers of the heart. If a physiological or pathological alteration was present to cause right ventricular hypertrophy, there would also be a certain degree of hypertrophy of that portion of the septum which forms the medial wall of the right ventricle.

TABLE 3—Ventricular Ratios, Experiment 1 (1956)

Value	RV/T	LV/T	S/T
5,000 FT.			
Mean	0.244	0.490	0.266
Range	0.210–0.265	0.429–0.545	0.267–0.361
Standard deviation	0.013	0.026	0.020
9,000 FT.			
Mean	0.269	0.462	0.270
Range	0.243–0.287	0.390–0.499	0.239–0.338
Standard deviation	0.012	0.030	0.026

At each altitude, values were obtained from 19 cattle.

Experimental Studies.—The fact that no clinical cases of high mountain disease developed in the cattle in the experimental groups was not unexpected. The normal incidence of the disease is approximately 0.5 to 1.0 per cent of animals residing above 7,000 ft. The total number of animals in the two experiments at 9,000 ft. was 40. This number is less than half of that needed to statistically expect a naturally developing case.

Experiment 1.—In experiment 1, there was a highly significant increase in the ratio of the right ventricle to the total ventricular weight, due to the effect of the altitude treatment alone. Here again is the

problem as to whether this is actual right ventricular hypertrophy or is only relative due to a decrease in the left ventricular and septal weights.

The animals in this experiment were of uniform breeding and weight and were randomly allotted to the treatment groups by weight. At the beginning of the experiment, there was no statistical difference in body weight. At the termination of the trial there was again no significant difference in body weight (table 2). In these animals, therefore, a statistical comparison of the actual weight of the ventricular components is valid.

Table 2 shows a highly significant increase in the weight of the right ventricle due to the effect of altitude, but no statistical difference in the weight of the left ventricle of the four treatment groups. The decrease in the weight of the septum, due to interaction of the main effects, is probably due to variation in dissection technique rather than to experimental treatment.

It is inferred from the experimental data that the increase in the RV/T ratio is due to the effect of altitude and reflects an actual increase in weight of the right ventricle; *i.e.*, right ventricular hypertrophy.

Experiment 2.—The results revealed a highly significant increase in the RV/T ratio under the effects of altitude (table 4). The LV/T ratio decreased with the same statistical probability and there was no significant alteration in the S/T ratio.

Again in this experiment, there was no significant difference in initial body weight of the animals in the respective treatment groups. At the termination of the experiment, however, there was a highly significant increase in body weight due to the effects of altitude. This alteration made invalid a comparison of the actual weight of the ventricular components.

In this experiment, the animals residing at high altitude were grazing on far superior pasture than were those at low altitude. This fact is believed to account for the difference in final body weight in this trial.

TABLE 4—Experiment 2, Analysis of Variance (1957)

Measurement	Key out	Effect	df	Probability
Initial body weight (lb.)	Mean	560.0		
	A	2.0	1	
	B	2.5	1	
	AB	−2.0	1	
	Error	.....	36	
Final body weight (lb.)	Mean	691.0		
	A	58.0	1	<.01
	B	−7.0	1	
	AB	−7.0	1	
	Error	.....	36	
RV/T	Mean	0.266		
	A	0.018	1	<.01
	B	−0.005	1	
	AB	−0.002	1	
	Error	.....	36	
LV/T	Mean	0.464		
	A	−0.014	1	<.05
	B	0.003	1	
	AB	−0.002	1	
	Error	.....	36	
S/T	Mean	0.270		
	A	−0.004	1	
	B	0.002	1	
	AB	0.004	1	
	Error	.....	36	

A = effect of altitude; B = effect of emboli; AB = effect of interaction of altitude and emboli.

TABLE 5—Ventricular Ratios, Experiment 2 (1957)

Value	RV/T	LV/T	S/T
5,000 Ft.			
Mean	0.258	0.471	0.272
Range	0.235–0.284	0.434–0.500	0.251–0.296
Standard deviation	0.003	0.007	0.019
9,000 Ft.			
Mean	0.275	0.456	0.268
Range	0.226–0.324	0.427–0.500	0.237–0.289
Standard deviation	0.025	0.017	0.016

At each altitude, values were obtained from 20 cattle.

Based on information obtained in experiment 1, where the final body weight was the same for all groups, it is concluded that the increase in the RV/T ratio is due to an increase in the right ventricular weight and not a decrease in weight of the other components.

The means for the RV/T ratio of both the high and low altitude animals were increased in experiment 2 as compared with experiment 1 (tables 3, 5). The animals used in experiment 2 came from an altitude of 2,500 to 3,500 ft., while those used in experiment 1 came from an altitude of approximately 5,000 ft. A postulation is made that the animals in the second experiment underwent greater altitude stress change, resulting in a mild right ventricular hypertrophy in both low- and high-altitude groups. The difference between the RV/T ratios in the high- and low-altitude groups in both experiments is approximately the same (0.025 in experiment 1 and 0.023 in experiment 2).

The study of hearts from cattle with clinical cases of high mountain disease reveals that there is a marked right ven-

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tricular hypertrophy accompanied by a moderate hypertrophy of the septum. These changes are compatible with the clinical and necropsy syndrome of congestive failure of the right heart that is seen in this disease.

From the designed experiments it may be concluded that residence at high altitude causes a mild, but statistically significant, hypertrophy of the right ventricle of the heart in cattle. This finding strengthens the premise that residency at high altitude is probably an important factor in the cause of high mountain disease of cattle.

Cardiac muscle, like skeletal muscle, hypertrophies when an increased work load is placed on it. The right ventricular hypertrophy seen with residence at high altitude must, therefore, be due to an increased work load on the right heart. The function of the right ventricle is to pump unoxygenated blood through the lungs where gaseous exchange takes place. The increased work load upon the right heart must, therefore, be due to some alteration in the pulmonary circulation with a resulting increase in resistance to flow.

Increased blood viscosity or volume, or both, are factors which might elicit this change. The polycythemia, which is generally accepted as accompanying acclimatization to high altitudes, could presumably cause an increase in blood viscosity. An increase in total blood volume has also been shown to accompany acclimatization to high altitudes.[16] These changes, however, should place an equal stress on both the right and left heart and cause bilateral changes. However, the changes appeared to be confined to the right ventricle. The possibility exists that the left ventricle has sufficient reserve strength that such a mild stimulus would not cause it to hypertrophy.

A more plausible explanation is that hypoxia experiments, it is possible that a atmospheres, causes an increase in the pulmonary resistance, an elevation in the pulmonary arterial pressure and, consequently, a greater work load on the right heart. The work of von Euler and Liljestrand [5] with cats and of Lewis and Gorlin [11] with dogs lends support to this hypothesis. While these investigators found elevated pulmonary arterial pressures in acute hypoxia experiments, it is possible that a chronic hypoxic stimulus could cause a mild increase in pulmonary vascular resistance and pressure, and thus account for the moderate right ventricular hypertrophy which occurred.

In addition, moderate vascular constriction, over and above that found at lower elevations, could stimulate the development of permanent anatomic changes in the pulmonary arteries and arterioles. Such changes could include medial hypertrophy, the laying down of medial collagen, and intimal proliferation.

An additional possibility exists wherein the effects of pulmonary hypertension increased blood viscosity, and increased blood volume, combine to increase the work load of the right side of the heart.

SUMMARY

1) A study was made to determine the location and degree of gross cardiac changes within the hearts of 41 cattle with clinical cases of high mountain disease; 39 cattle residing at high altitudes; and 39 cattle residing at low altitudes.

2) Study of hearts from animals with clinical cases of high mountain disease revealed a marked right ventricular hypertrophy with mild hypertrophy of the septum.

3) Experimentation showed that continuous residence at high altitude caused mild, but statistically significant, hypertrophy of the right ventricle.

4) Chronic hypoxia is postulated to be a causative factor in the development of the right ventricular hypertrophy.

5) In the quantities given, showers of autogenous, fibrinous emboli into the pulmonary vascular system caused no significant cardiac hypertrophy.

References

[1] Atland, P. D., and Highman, B.: Acclimatization Response of Rats to Discontinuous Exposures to Simulated High Altitudes. Am. J. Physiol., *167*, (1951): 261–267.

[2] Caparó, A. C.: Policitemia y Mal de Montana en Corderos. A thesis (December, 1950), Lima, Peru.

[3] Caparó, A. C., Copaira, M., and de la Vega, E.: Mal de Montana Coronico en Vacunos. Anales Fac. med., *38*, (1955): 1–10.

[4] Castro, J. D. de, and Iglesias, B.: Preliminary Report on Anatomic Studies at High Altitudes. U.S.A.F. School Aviation Med., Report No. 55–97, (1956): 6 p.

[5] Euler, U. S. v., and Liljestrand, G.: Observations on the Pulmonary Arterial Blood Pressure in the Cat. Acta physiol. scandinav., *12*, (1946): 301–320.

[6] Glover, G. H., and Newsom, I. E.: Brisket Disease. Colorado Agric. Exper. Station Bull. 204, Jan., 1915.

[7] Glover, G. H., and Newsom, I. E.: Brisket Disease. Colorado Agric. Exper. Station Bull. 229, May, 1917.

[8] Glover, G. H., and Newsom, I. E.: Further Studies on Brisket Disease. J. Agric. Res., *15*, (1918): 409–413.

[9] Graybiel, A., Patterson, J. L., and Houston, C. S.: Changes in Heart Size in Man During Partial Acclimatization to Simulated High Altitudes. Circulation, *1*, (1950): 991–999.

[10] Kerwin, A. J.: Observations on Heart Size of Natives Living at High Altitudes. Am. Heart J., *28*, (1944): 69–80.

[11] Lewis, B. M., and Gorlin, R.: Effects of Hypoxia on Pulmonary Circulation of the Dog. Am. J. Physiol., *170*, (1925): 574–587.

[12] Moore, C. R., and Price, D.: A Study at High Altitude of Reproduction, Growth, Sexual Maturity, and Organ Weights. J. Exptl. Zool., *108*, (1948): 171–216.

[13] Newsom, I. E.: Cardiac Insufficiency at High Altitude. Am. J. Vet. Med., *10*, (1915): 890–893.

[14] Penaloza, D.: Electrocardiographic Observations on Ten Subjects at Sea Level and During One Year of Residence at High Altitude. U.S.A.F. School Aviation Med., Report No. 56–98, (1956): 12 p.

[15] Pierson, R. E., and Jensen, R.: Brisket Disease. In "Diseases of Cattle." American Veterinary Publications, Evanston, Ill., 1956.

[16] Riessmann, K. R.: The Blood Volume in the Dog During Altitude Acclimatization. U.S.A.F. School Aviation Med., Report No. 1, (1951): 8 p.

[17] Rotta, A.: Physiologic Condition of the Heart in Natives of High Altitudes. Am. Heart J., *33*, (1947): 669–676.

[18] Ryff, J. F.: Brisket Disease Syndrome. J.A.V.M.A., *131*, (Nov. 1, 1957): 425–430.

[19] Sera, R., and Jensen, R.: Unpublished data, Colorado State University, Fort Collins, 1955.

[20] Stickney, J. C., and Van Liere, E. J.: Acclimatization to Low Oxygen Tension. Physiol. Rev., *33*, (1953): 13–34.

[21] Tappan, D. V., and Reynafarje, B.: Tissue Pigment Studies in Altitude Adaptation. U.S.A.F. School Aviation Med., Report No. 56-97, (1956): 8 p.

[22] Van Liere, E. J.: The Effect of Prolonged Anoxemia on the Heart and Spleen in the Mammal. Am. J. Physiol., *116*, (1936): 290–294.

[23] Van Liere, E. J., and Stickney, J. C.: Organ Weight-Body Weight Ratios in Dogs Following Exposure to Discontinuous Anoxia. J. Aviation Med., *14*, (1943): 194–199.

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Sodium Metabisulfite and Sulfur Dioxide Gas (Silage Preservative) Poisoning in Cattle

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THE BENEFICIAL EFFECTS of sodium metabisulfite as a silage preservative have been reported.[1,3,4,8,9] The metabisulfite method of silage preservation has been extensively used, and there have been no confirmed cases of intoxication associated with the feeding of this silage. However, there have been occasional obscure cases of intoxication in which the feeding of metabisulfite silage was suggested as a possible cause.[6]

Studies on the toxicity of metabisulfite and sulfur dioxide gas for cattle and observations on the effects of feeding metabisulfite silage to cattle over a period of several years are reported in this paper.

MATERIALS AND METHODS

Sodium metabisulfite was given twice daily to fistulated dairy cattle immediately after it was prepared as a water solution. Only the measured amount of metabisulfite introduced into the rumen through the fistula was used in calculating intake. One half of the daily dose was given in the morning and the other half in the late afternoon. Small additional but variable amounts of metabisulfite were consumed when some of the animals were fed preserved silage, but no attempt was made to use these amounts in calculating the intake. A small cylinder of sulfur dioxide was used to introduce this gas constantly through a tube in the fistula. The flow was regulated by a needle valve and cylinder weights were recorded in order to determine the amount of gas introduced during each 24-hour period.

Information on experimental animals is given (table 1). All animals were clinically examined daily.

Blood was collected from the jugular vein of each animal and smears for differential leukocyte determinations were made within 15 minutes after collection. Sodium fluoride was used as the anticoagulant in samples collected for blood glucose determinations, and potassium oxalate was used in the samples collected for blood urea nitrogen determinations. Potassium and ammonium oxalated blood was used for all other samples.

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The following procedures were used for hematological and biochemical determinations: Wright's stain for differential leukocyte counts,[7] Peter and Van Slyke's method for blood glucose,[2,11] and Van Slyke and Cullen's method for blood urea nitrogen.[11] Sections were taken at necropsy and stained with hematoxylin and eosin.

Base-line clinical, hematological, and biochemical data were collected for at least one week on animals B121, B190, B230, and B262 prior to giving the preservatives.

EXPERIMENTAL PROCEDURE AND CLINICAL RESULTS

Experiment 1.—Cows B164 and B145, on a ration of clover silage and concentrate, were given bisulfite at the rate of 80 Gm. per day. The rate was increased 40 Gm. every two days. On the sixth day, both cows quit eating and bisulfite feeding was discontinued. Appetites gradually returned and, six days after the experiment was stopped, both cows were on full feed. Three days after the cows returned to full feed, they were regarded as being normal. A new trial was then initiated at the level of 80 Gm. per day. Only the amounts of metabisulfite given in the second trial were used in calculating the total intake on this experiment.

Cow B164, which was in the third month of gestation at the start of the experiment, was given 80 Gm. per day for 180 days, at which time she calved normally. Neither the cow nor the calf showed any abnormalities at any time.

Cow B145 was given metabisulfite at the rate of 80 Gm. per day for the first week, 120 Gm. per day the second week, and 160 Gm. per day the third week. On the eighteenth day of the experiment, she was unable to stand and showed occasional convulsions. By the twentieth day, there was complete anorexia and the cow was killed for necropsy.

Experiment 2.—Heifers B121 and B190 were used in experiment 2. At the beginning of the experiment, heifer B121 was

fed preserved grass silage ad libitum. As the dosage of metabisulfite increased, the silage intake declined, and it was necessary to add hay and grain to the ration. Heifer B190 was given only hay and grain throughout the experiment.

Heifer B121 was started at a daily dosage of 80 Gm. of metabisulfite. The daily dosage was increased to 120 Gm. for the second week, 160 Gm. for the third week, 200 Gm. during the fourth and fifth weeks, and 240 Gm. per day for three days of the sixth week, when she died. The first signs of anorexia occurred during the second week, when the dosage of metabisulfite was 120 Gm. per day. Other signs of illness in the order of their appearance were: constipation, mild depression, dyspnea, vomiting, excessive salivation, diarrhea, severe dysentery, and terminal depression.

Heifer B190 was started on a daily dosage of 80 Gm. of metabisulfite, the dose was increased to 120 Gm. for the second week, and 160 Gm. per day was given for two days of the third week, at which time she died. This animal exhibited a marked decline in hay and grain consumption starting on the third day of the experiment and continuing until the twelfth day, when anorexia was complete. Other clinical signs observed were occasional coughing, constipation, and severe terminal dysentery.

Experiment 3.—Sulfur dioxide gas was introduced through rumen fistulas in steers B230 and B262.

In steer B230, the gas was released at a point well above the liquid contents of the rumen. The amount of gas introduced was 50 Gm. per day for the first week, 162 Gm. per day for the second week, and 255 Gm. per day for two days of the third week, when the steer died.

A temperature of 104.8 F. was observed on the fifth day and the first signs of anorexia occurred on the tenth day. A heavy mucopurulent nasal discharge, followed by diarrhea, constipation, and coughing, was observed on the ninth day and continued until the animal died.

Since considerable necrosis of the dorsal rumen wall of steer B230 occurred as a result of direct contact with sulfur dioxide gas, in steer B262 the gas was introduced into the liquid portion of the rumen contents. This steer was given 53 Gm. per day the first week; 73 Gm. per day the second week; 145 Gm. per day the third week; and 47 Gm. on the first day of the fourth week, when death occurred. Anorexia began on the fourteenth day. Other clinical signs were similar to those observed in steer B230, except for terminal nystagmus and severe depression.

TABLE 1—Results from Feeding Sodium Metabisulfite and Sulfur Dioxide Gas to Fistulated Cattle

No. and breed	Sex and age	Estimated body weight (lb.)	Estimated body weight lost (lb.)	No. experiment days	Total bisulfite or SO_2 (Gm.)	Results
B164 Guernsey	Cow adult	1,200	None	180	14,400	Normal
B145 Holstein	Cow adult	1,100	.....	20	2,360	Death
B121 Ayrshire	Heifer 16 months	850	250–300	37	6,040	Death
B190 Shorthorn	Heifer 16 months	600	200	16	1,720	Death
B230 Jersey	Steer 14 months	600	250	16	1,995 (SO_2)	Death
B262 Holstein	Steer 9 months	600	200	21	1,944 (SO_2)	Death

100 lb. of well-preserved silage contains approximately 45 Gm. sulfite.

General Results

The clinical course was 37, 16, 16, and 21 days for animals B121, B190, B230, and B262, respectively (table 1). The onset and severity of the signs observed were closely correlated with the amounts and rates of administration of the preservatives. Anorexia and severe body weight loss were the predominating signs of illness. Constipation, diarrhea, depression, and dysentery were common signs. Anorexia was observed at three and eight days, respectively, in the 2 animals given sodium metabisulfite, and at 10 and 14 days in the 2 steers given sulfur dioxide gas. Anorexia in heifer B190 was complete for six days.

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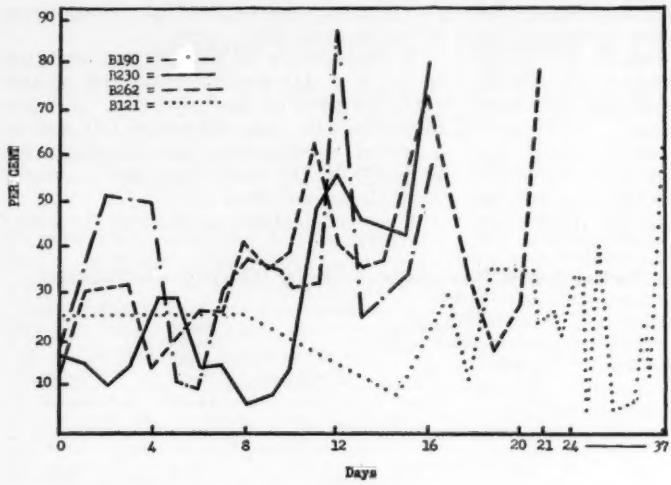


Graph 1—Changes in neutrophil percentage during the course of sodium metabisulfite and sulfur dioxide gas intoxication.

Depression was more severe and constant in animals given metabisulfite, but discharge from the external nares was not observed in these animals. One of the animals given sulfur dioxide gas had considerable heavy mucopurulent discharge, and the other a thick mucous nasal discharge. Coughing varied from mild to moderate and was inconsistent and sporadic.

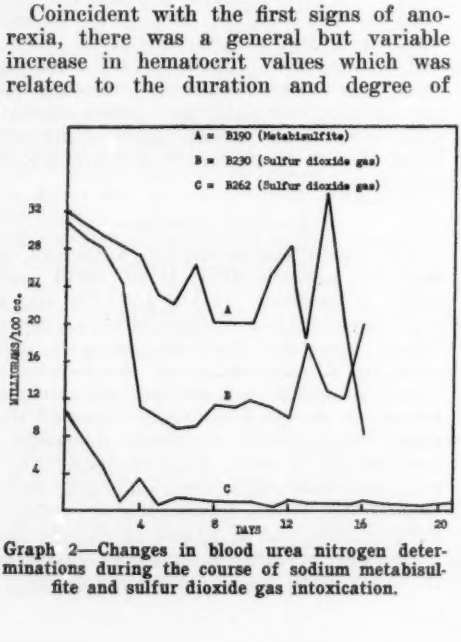
HEMATOLOGICAL OBSERVATIONS

Coincident with the first signs of anorexia, there was a general but variable increase in hematocrit values which was related to the duration and degree of anorexia. Erythrocyte counts tended to parallel the hematocrit values. The mean corpuscular hemoglobin concentration gradually decreased, except for minor fluctuations in all 4 animals.

Leukocyte counts generally ranged from 9,500 to 15,000/cmm., but several counts of 21,000 to 35,000 were observed. The higher counts were one- or two-day peaks occurring in the latter half of the experimental period.

The usual range of icterus index was 10.0 to 15.0 during the last five to ten days of the experimental periods. In 1 animal, it reached 21.1.

A constant shifting of the differential leukocyte counts occurred when the rate of preservative administration was at the higher levels. The more pronounced changing occurred in the neutrophils with a corresponding shift of the large and small lymphocytes (graph 1). An occasional mild to moderate single-day increase occurred in the eosinophils, monocytes, and basophils but, in general, no significant change occurred.



Graph 2—Changes in blood urea nitrogen determinations during the course of sodium metabisulfite and sulfur dioxide gas intoxication.

BIOCHEMICAL OBSERVATIONS

No significant change in blood glucose levels was observed. Blood urea nitrogen values decreased significantly in all animals (graph 2).

Residual sulfite of the rumen contents, determined by the method of Nichols and Reed,[10] showed that added sulfite disappeared rapidly until the dosage level of

200 Gm. per day was reached (graph 3). At this point, the residual sulfite of the rumen contents was extremely high; during the two days preceding death, the level was so high that an accurate determination could not be made.

NECROPSY OBSERVATIONS

Rumen.—Rumen lesions varied with the preservative given. General thickening of the rumen wall and mild, subserous, suffusion hemorrhage occurred in the 2 animals given sulfur dioxide gas. In all 5 animals which died, there was a mild to moderate congestion of the mucosa. In 1 animal, the gas directed toward the dorsocaudal portion of the rumen produced a severely thickened rumen wall over an area of 10 square inches. Marked suffusion hemorrhage occurred in the serosa, while a ¼-inch-thick membranous exfoliation resulted on the mucosal surface.

Microscopically, congested portions of the rumen showed severe congestion of the lamina propria as well as edema. There were a few focal areas of hemorrhage, erosions and leukocyte infiltrations of the tips of the papillae. In the more severely thickened portions of the rumen wall, there was complete structural disorganization. Most of the mucosa was eroded; considerable leukocyte infiltration and marked increase of connective tissue were observed.

Reticulum.—Moderate congestion occurred in 4 of the 5 cattle. Upon microscopic observation, there were variable erosions and leukocyte infiltrations of the tips of the papillae. In 1 animal, mild coagulation necrosis occurred under the cornified stratified squamous epithelium.

Omasum.—Gross and microscopic lesions similar to those in the reticulum were observed. In addition, in 1 animal the contents were fluid, while in another there was localized necrosis of the "leaves."

Abomasum.—One animal on each preservative developed acute hemorrhagic gastritis. The animal given sulfur dioxide gas had numerous bleeding points, and the abomasal contents were chocolate colored. The other 3 animals showed mild to moderate congestion of the mucosa.

Histologically, there was usually some erosion, leukocytic infiltration, congestion, and hemorrhage of the mucosa. In the animals with more severe cases, there was severe edema of the submucosa and tunica muscularis.

Small Intestines.—Gross pathological changes varied from none to acute hemorrhagic enteritis and corresponded with the degree of changes in the other organs. These varied with the type and total amount of preservative administered, and the duration of administration. Animals B145 and B121 showed no gross lesions, B190 and B230 showed a few petechial hemorrhages and marked catarrhal enteritis, and B262 had marked hemorrhagic enteritis of the duodenum that changed into marked catarrhal enteritis.

Histopathologically, there were varying degrees of hyperemia, edema, and cellular infiltration of the propria. The villi were swollen. In animals with more severe cases, the inflammatory reaction extended to the submucosa, with severe mucous degeneration in Lieberkühn's glands to the extent that few of the gland cells remained intact. The usual superficial effects of desquamation, erosion and, in a few cases, leukocytic infiltration were observed.

Large Intestines.—Grossly, acute catarrhal enteritis was observed in steer B262, whereas only a few sporadic petechial hemorrhages and hyperemia were observed in the other animals.

Microscopically, only the superficial effects of desquamation, erosion, hyperemia, and occasional petechial hemorrhages were found.

Liver and Gallbladder.—Grossly, the liver showed either mild congestion and a few localized areas of fatty degeneration or no pathological changes. The variation was associated with the severity and duration of illness and the total amount of preservative administered. The gallbladder of steer B262 showed several ecchymotic hemorrhages in the mucosa and thickened bile. The bile was also thickened in heifer B121.

Microscopically, the livers of animals B121 and B262 showed focal areas of coagulation necrosis, cloudy swelling, and fatty degeneration as compared with severe fatty degeneration, coagulation necrosis, and congestion in heifer B190.

Spleen and Lymph Nodes.—A few petechial hemorrhages were observed in the spleen of steer B262; in heifer B121, the spleen capsule was thickened and the mesenteric lymph nodes were enlarged and edematous. In cow B145, the parotid and submandibular lymph nodes were enlarged and edematous.

Kidney.—Mild to moderate swelling oc-

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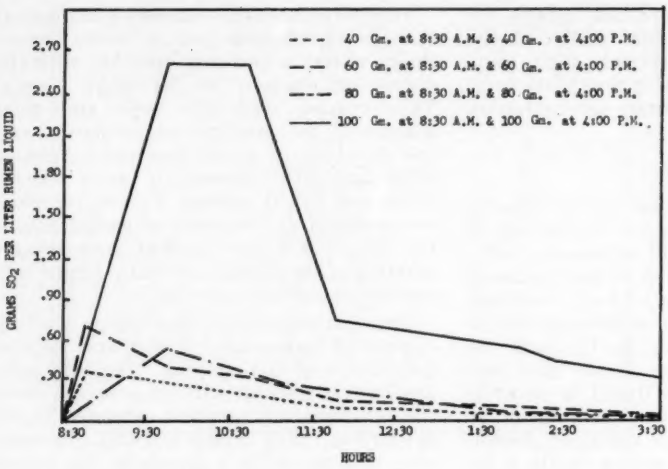


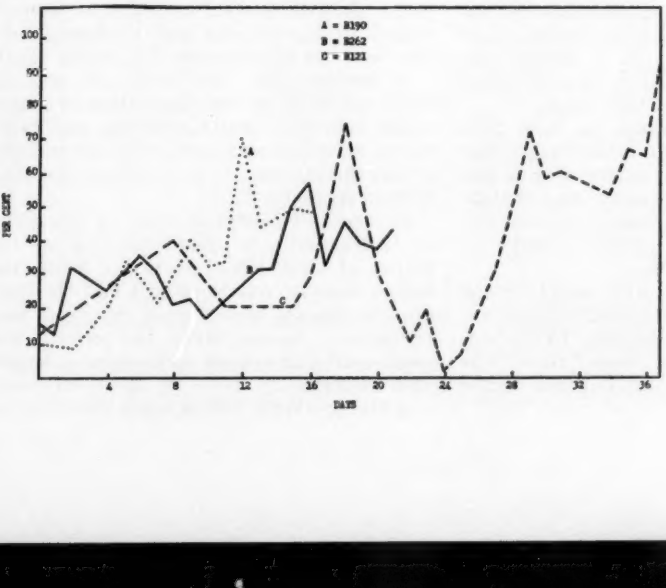
Graph 3—Rate of disappearance of sodium metabisulfite in rumen contents of heifer B121 when added through a fistula.

curred in the kidneys of 3 of the 5 animals, with mild to moderate congestion in 2 of the cattle. The kidneys appeared shrunken in 1 animal.

Microscopically, the kidneys of animals B121, B190, B230, and B262 showed moderate to severe nephrosis with some moderate cystic dilatation of the convoluted tubules. Occasionally leukocytic infiltrations were seen.

Respiratory System.—In 3 of the animals, the lungs were mildly to moderately emphysematous. In heifer B121, there was marked emphysema, numerous subpleural petechial hemorhages, and a small area of acute bronchopneumonia in the dependent portion of the apical lobe of the right lung. In 2 animals, there was severe hemorrhage and edema of the larynx, while in the other 3 animals necropsied the larynx was apparently normal. Animals B121, B190, and B262 had severe petechial ecchymotic and suffusion hemorrhages along the ventral wall of the cranial portion of the trachea (fig. 1). In addition, there was moderate submucosal edema of the trachea. Only a mild to moderate congestion was observed in 1 of the remaining 2 animals.

Cardiovascular System.—Steer B262 had a moderate number of petechial, ecchymotic, and suffusion hemorrhages of the myocardium and endocardium. In the other 4 animals, there were only a few petechial and occasionally mild, diffuse suffusion-type hemorrhages. Microscopically, only hemorrhages were observed.



Graph 4—Changes of percentage of degenerating leukocytes in 3 cattle during the course of sodium metabisulfite and sulfur dioxide gas intoxication. Curve A is also typical for steer B230.

Central Nervous System.—In animals B145 and B121, which showed clinical central nervous system disturbances, there was a moderate congestion of the leptomeninges, and apparently an increase in quantity of gelatinous, cerebrospinal fluid. In the other 3 animals, there was a mild congestion and edema of the leptomeninges, with some apparent increase in cerebrospinal fluid.

Microscopically, only mild to moderate inflammatory changes of the leptomeninges were observed.

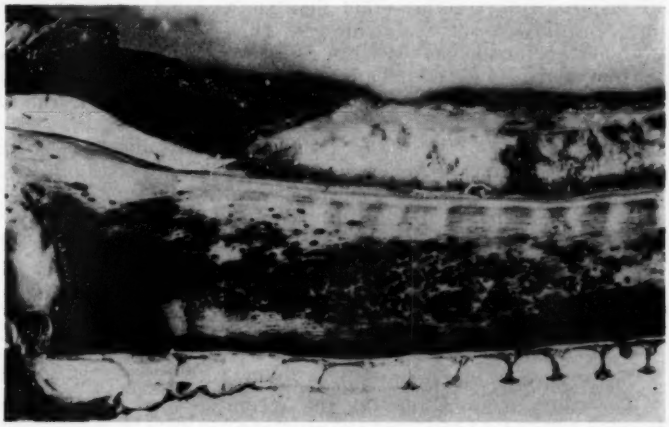


Fig. 1—Hemorrhages in the mucosa of the ventral wall of the cranial part of the trachea of steer B262 following the introduction of sulfur dioxide gas into the rumen contents.

Discussion

Severe intoxication in cattle from feeding silage preserved with these agents seems highly improbable because of the resultant anorexia. Furthermore, the animal's appetite for preserved silage was decreased prior to general anorexia. The dosage which produced anorexia was within the range of 80 to 160 Gm. of metabisulfite or sulfur dioxide gas per day.

Nearly all of the 1,000 to 1,500 tons of grass silage produced annually at the university is treated with metabisulfite. Each year, for the past five years, approximately 600 cattle have been fed preserved silage with no apparent harmful effects.

Alderman *et al.*[1] reported that 48 samples of well-preserved, metabisulfite-treated silage showed an average residual sulfite content of 0.295 per cent in the dry matter. The mean value was the equivalent of 840 p.p.m. in the fresh silage, and represented only 25 per cent of the sodium metabisulfite originally added. A well-preserved silage, made from forage treated with sodium metabisulfite at the recommended rate of 8 lb. per ton of fresh forage, would contain approximately 2 lb. of residual sulfite per ton. An animal eating 100 lb. of this silage per day would consume approximately 45 Gm. of sulfite per day, or about half as much as cow B164 was given each day for six months with no adverse effects. Since anorexia occurs when a greater concentration of the agent is given, it appears that an animal can obtain toxic or lethal amounts only by forced feeding.

Sodium metabisulfite and sulfur dioxide gas are inhibitors of bacteria and certain enzyme systems. It is logical to assume that these preservatives would have a depressing effect on the microorganisms of the rumen. However, cow B164 received 80 Gm. of metabisulfite per day for six months and remained apparently normal, indicating that this effect may not be significant in the cow. The evidence obtained in rumen residual bisulfite determinations (graph 3) shows that when as much as 160 Gm. of metabisulfite per day is given the disappearance from the rumen is fairly rapid. At a level of 200 Gm. per day there was a bisulfite saturation, but the cow did not die until 15 days later.

The consistent and immediate drop in blood urea nitrogen in animals B190, B230, and B262 suggests that this may be a constant feature of metabisulfite intoxication. Since urea is the most important nitrogenous waste product, a disturbance in the

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nitrogen balance of the body probably occurs.[5]

Blood glucose values were quite variable and erratic and, consequently, not significant.

The only fairly consistent lesion produced was the hemorrhagic trachea. Since this lesion occurred in 2 of 3 animals given metabisulfite, it was believed to be caused by inspired rumen gases (other than methane), possibly by sulfur dioxide gas. However, sulfur dioxide gas placed in the dorsal portion of the rumen of steer B230 resulted in only a mild to moderate congestion of the trachea. Furthermore, when the gas was delivered into the liquid contents of the rumen of steer B262, severe tracheal lesions were observed. If the tracheal lesions result from sulfur dioxide, they are not caused by the gas per se. The ventral location of the lesions may indicate that it was due to a condensation of inspired rumen gas into moisture droplets.

SUMMARY

Cattle eating 100 lb. of silage treated with sodium metabisulfite at the recommended rate would consume approximately 45 Gm. of sulfite per day. One pregnant cow was given about twice this amount daily for 180 days with no deleterious effects. The 600 cattle maintained by the university have been fed metabisulfite-treated silage for more than five years with no deleterious effects.

When massive doses of sodium metabisulfite or sulphur dioxide gas were administered through rumen fistulas to 5 cattle, they died in 16 to 37 days. Anorexia was a consistently observed sign of the intoxication. The pathological changes varied considerably in each animal and with the preservative given. In general, the more severe tissue changes were confined to the first portion of the digestive tract. The most consistent lesions were in the larynx and in the mucosa of the ventral wall of the trachea.

There was no significant change in blood glucose, but blood urea nitrogen determinations in 3 animals revealed a definite drop in blood levels during the course of the experiment. Hematological data were highly variable.

A residual bisulfite determination on the rumen contents of 1 animal indicated that a strong homeostases body function existed until the animal apparently became saturated with the preservative. Saturation occurred when the daily dose of metabisulfite reached 200 Gm. However, the animal did not die until 15 days later.

References

[1] Alderman, G., Cowan, R. L., Bratzler, J. W., and Swift, R. W.: Some Chemical Characteristics of Grass and Legume Silage Made with Sodium Metabisulfites. J. Dai. Sci., *38*, (1955): 805–810.

[2] Benedict, S. R.: The Analysis of Whole Blood. II. The Determination of Sugar and Saccharoids. J. Biol. Chem., *92*, (1931): 141–159.

[3] Bratzler, J. W., Cowan, R. L., and Swift, R. W.: Preservation of Grass Silage with Sodium Metabisulfite. Pennsylvania Agric. Exper. Station Bull. 597, 1955.

[4] Cowan, R. L., Bratzler, J. W., and Swift, R. W.: Grass Silage Preservation with Sodium Bisulfite. Pennsylvania Agric. Exper. Station Progress Report 99, 1953.

[5] Dukes, H. H.: The Physiology of Domestic Animals. 7th ed. Comstock Publishing Associates, Ithaca, N.Y., (1955): 558.

[6] Elliott, George A.: Focal Necrosis in the Cerebral Cortex of the Bovine, a Histopathologic Study. University of Pennsylvania Vet. Exten. Quart. No. 150, (1958): 75–92.

[7] Gradwol, R. B. H.: Clinical Laboratory Methods and Diagnosis. 4th ed. C. V. Mosby Co., St. Louis (1948): 535.

[8] Knodt, C. B., Skaggs, S. R., and Williams, P. S.: Sulfur Dioxide for Preservation of Forage. Pennsylvania Agric. Exper. Station Bull. 552, 1952.

[9] MacPherson, H. T., Wylan, C. B., and Ramstad, S.: Changes in Carbohydrate, Nitrogen and Organic Acid Distribution in Grass Preserved with Sodium Metabisulfite. J. Sci. Food & Agric., *8*, (1957): 737.

[10] Nichols, P. F., and Reed, H. M.: Distillation Methods for the Determination of Sulfur Dioxide. Indust. Engineer Chem. Anal. ed., *4* (1932): 79.

[11] Peters, J. P., and Van Slyke, D. D.: Quantitative Clinical Chemistry. 2nd. ed., vol. 11. Williams & Wilkins Co., Baltimore, 1946.

The Effects of Four Synthetic Corticosteroids on Leukocytes, Blood Glucose, and Plasma Sodium and Potassium in the Cow

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IN RECENT YEARS, glucocorticoid therapy for ketosis in dairy cattle has become widely used. Its advantages lie in its ease of administration and prolonged blood glucose-elevating properties, when compared with many treatments currently employed. The synthesis of more potent corticoids will decrease dosage required, thereby lowering the cost of treatment. Many investigators have cited the various actions of these highly potent synthetic corticoids in laboratory animals, but less is known of their actions in cattle.

REVIEW OF LITERATURE

Using intact rats, Perlman and Tolksdorf [11] reported 1-dehydrocortisone (prednisone, Δ' E) and 1-dehydrohydrocortisone (prednisolone, Δ' F) to be three to four times more active than cortisone (E) or hydrocortisone (F) when compared on the basis of eosinopenic response, liver glycogen deposition, and thymus involution following intramuscular injection of these compounds. West [15] found Δ' E and Δ' F to be five times as potent as E in their hyperglycemic effects in human subjects, whereas their sodium-retaining ability was not increased above that of the parent compound in adrenalectomized rats [11] or human beings.[1]

It has also been shown that when hydrocortisone bears chlorine or fluorine in the 9-*alpha* position, it not only becomes more potent than hydrocortisone in carbohydrate and eosinopenic activity,[6] but also becomes more potent that desoxycorticosterone in sodium-retaining activity.[6, 14] Stafford *et al.*,[13] using adrenalectomized rats, compared the glucocorticoid and mineralocorticoid activity of 9-*alpha* fluorohydrocortisone (9 α FF) and 1 dehydro 9-*alpha* fluorohydrocortisone (9 α fluoroprednisolone, Δ' 9 α FF), and reported Δ' 9α FF to be four times as potent as 9 α FF in the liver glycogen deposition test but no more potent than 9 α FF in the sodium retention test. Lyster *et al.*,[8] also using adrenalectomized rats, reported 6 methyl 1-dehydrohydrocortisone (methylprednisolone, 6M Δ' F) to be 3 times as potent as Δ' F in increasing liver glycogen deposition, although these compounds were similar in their failure to induce sodium retention.

Because of the profound effects of these compounds in laboratory animals, an experiment was designed to determine the effects of two dosages of four steroids on blood glucose, leukocytes, and plasma sodium and potassium in the cow. The compounds selected represent an unsaturated steroid (Δ' F), a halogenated steroid (9 α FF), an unsaturated-halogenated steroid (Δ' 9 α FF), and an unsaturated-methylated steroid (6M Δ' F).

MATERIALS AND METHODS

Eight multiparous, nonpregnant, lactating Holstein-Friesian cows, grazing on Bermuda grass pasture and averaging 940 lb. of body weight, were randomly assigned to one of the following treatments: 50 mg. of Δ' F; 100 mg. of Δ' F; 25 mg. of 9 α FF; 100 mg. of 9 α FF; 25 mg. of Δ' 9 α FF; 100 mg. of Δ' 9 α FF; 25 mg. of 6M Δ' F; or 100 mg. of 6M Δ' F. The compounds were in aqueous suspension and were the alcoholic form, except for methylprednisolone acetate. All compounds were injected intramuscularly (i.m.) in the gluteal region. Blood samples were drawn 72, 24, and 0 hours prior to the injection to obtain normal values, and at 24, 48, 96, and 144 hours postinjection to determine treatment effects. This procedure was replicated six times at two-week intervals.

Blood glucose, plasma sodium and potassium, total leukocytes, and differential leukocytes were determined for each blood sample. Blood glucose was determined by the method described by Nelson.[10] Plasma sodium and potassium were determined with a flame photometer* using a 1,000 p.p.m. lithium internal standard. The data were subjected to a Student's "t" test to determine differences caused by treatments when compared with the 0-hour control samples.

RESULTS

The average blood glucose values prior to and following the i.m. injection of the various compounds are shown (table 1) and depicted graphically (graphs 1, 2). Fifty and 100 mg. of Δ' F produced significant increases in blood glucose values

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* Perkin-Elmer Flame Photometer, manufactured by Perkin-Elmer Corp., Norwalk, Conn.

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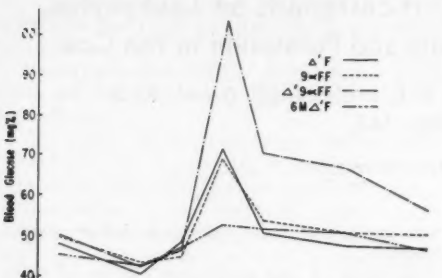
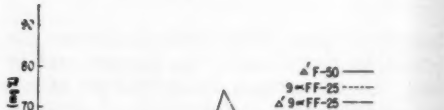


Graph 1—Average blood glucose values prior to and following the administration of 100 mg. of Δ′F, 9α FF, Δ′9α FF, and 6M Δ′F.

at 24 hours postinjection, when compared with those obtained at the time of injection. However, at 48 hours postinjection, these values had returned to essentially preinjection levels. At the 25-mg. dosage, 9α FF did not produce a significant change in blood glucose whereas, at the 100-mg. level, this compound produced highly significant increases. This elevation was transitory, however, since 48-hour blood samples contained nearly normal quantities of glucose. The most potent compound with respect to its ability to elevate blood glucose was Δ′9α FF. Both 25- and 100-mg. dosages of this material produced highly significant increases 24 and 48 hours postinjection and, at 96 hours, the higher dosage still maintained highly significant values. The methylated corticoid did not alter blood glucose values significantly at either dosage. The maximum blood glucose elevation following the administration of the various compounds is shown graphically (graph 3).



Graph 2—Average blood glucose values prior to and following the administration of 50 mg. of Δ′F and 25 mg. of 9α FF, Δ′9α FF, and 6M Δ′F.

At both dosages, Δ′F and Δ′9α FF produced significant increases in leukocyte numbers 24 hours postinjection (table 2). One hundred milligrams of Δ′9α FF was more prolonged in this respect, since 96-hour samples contained significantly more leukocytes when compared to preinjection blood samples.

To determine whether the increase in leukocyte numbers was due to an increase in all types of white cells or to one or more

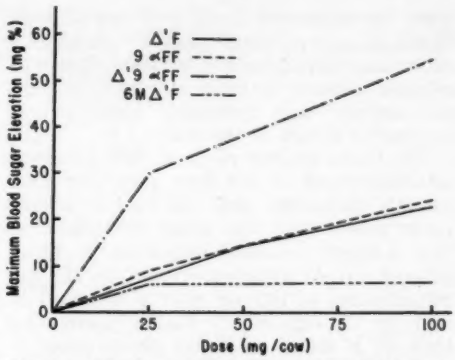
TABLE 1—Average Blood Glucose (mg./100 cc.) and Plasma Sodium and Potassium (mg./100 cc.) Prior to and Following the Administration of Δ′F, 9α FF, Δ′9α FF, and 6M Δ′F

Hours post-injection	Fraction	Treatment and dosage (mg.)							
		Δ′F-50	Δ′F-100	9α FF-25	9α FF-100	Δ′9α FF-25	Δ′9α FF-100	6M Δ′F-25	6M Δ′F-100
−72	Glucose	48.0	48.0	48.0	49.8	48.6	50.0	47.5	45.2
	Na	312.0	317.0	318.0	316.0	318.0	313.0	321.0	320.0
	K	17.7	17.0	17.3	17.4	17.4	16.4	17.7	17.2
−24	Glucose	44.5	40.3	43.2	43.2	43.4	42.5	43.7	42.7
	Na	315.0	305.0	310.0	311.0	311.0	311.0	311.0	305.0
	K	14.5	17.0	17.4	18.1	17.6	17.8	16.9	17.4
0	Glucose	47.5	48.3	47.8	44.8	43.8	47.0	46.5	46.0
	Na	314.0	315.0	318.0	314.0	320.0	314.0	310.0	312.0
	K	17.6	18.2	17.8	17.1	16.6	17.8	17.4	17.7
24	Glucose	61.8*	71.3*	57.3	68.8**	73.7†	103.2**	52.8	52.5
	Na	316.0	317.0	315.0	320.0	321.0	318.0	312.0	320.0
	K	18.2	18.3	17.6	16.5	17.3	17.2	17.5	18.8
48	Glucose	51.2	50.8	49.3	53.5	54.1†	70.3†	50.4	51.1
	Na	333.0	321.0	319.0	316.0	316.0	320.0	306.0	318.0
	K	18.3	18.9	17.3	16.9	18.2	16.4	18.4	18.3
96	Glucose	47.8	47.3	48.2	50.3	49.1	66.8†	51.0	50.3
	Na	312.0	309.0	308.0	310.0	318.0	309.0	306.0	302.0
	K	16.7	17.7	17.7	17.5	17.3	15.8	18.4	17.6
144	Glucose	46.8	46.5	50.2	50.0	46.8	56.0	49.5	46.1
	Na	311.0	314.0	315.0	317.0	316.0	318.0	311.0	312.0
	K	17.7	17.5	16.3	16.0	17.6	16.3	17.7	16.5

* P = 0.05; † P = 0.01; ** P = 0.001.

specific cell types, differential counts were performed. These values, and total leukocyte numbers, were used to compute the average total number of each type of white cell per cubic millimeter of blood. It was evident (table 2) that circulating lymphocyte numbers were not altered by any of the treatments. The average numbers of eosinophils prior to and following the various treatments are also shown (table 2). Although all compounds produced what appeared to be various degrees of eosinopenia, with the exception of 6M Δ'F, at no time were these changes significant. All compounds caused increases in neutrophil numbers (table 2) at 24 hours postinjection, although the changes produced by the lower dosage of 9 α FF and 6M Δ'F were not significant. The most prolonged effect on neutrophil numbers was caused by 100 mg. of Δ'9 α FF, since significant increases were still evident 96 hours after treatment.

Average plasma sodium and potassium levels before and after treatment are indicated (table 1). No changes in either sodium or potassium values were evident in postinjection blood samples when compared with preinjection samples. At no time did any of the animals show objective clinical signs, such as anorexia and muscular weakness, which are usually associated with hypokalemia.



Graph 3—Maximum blood glucose elevation following the administration of four hydrocortisone analogues.

DISCUSSION

The increased potency of these hydrocortisone analogues over the parent compound may be due to their resistance to metabolic degradation. Collins *et al.*[3] found the half-life of Δ'F to be 1.37 times that of F and suggested that, since it was more slowly metabolized, this may explain, in

TABLE 2—Average White Blood Cells (thousands/cmm.) Prior to and Following the Administration of Four Synthetic Corticoids at Two Dosages to Dairy Cattle

Hours post-injection	Cell	Treatment and dosage (mg.)							
		Δ' F-50	Δ' F-100	9 α FF-25	9 α FF-100	Δ' 9 α FF-25	Δ' 9 α FF-100	6M Δ' F-25	6M Δ' F-100
−72	Leukocyte	9.9	7.8	7.4	7.8	8.4	7.9	8.7	8.3
	Lymphocyte	5.4	4.7	4.2	4.1	4.0	4.5	4.5	5.0
	Neutrophil	3.9	2.3	2.7	2.9	3.2	2.5	2.9	2.5
	Eosinophil	0.42	0.53	0.38	0.47	0.99	0.59	0.91	0.65
−24	Leukocyte	8.4	8.0	8.2	9.1	9.3	8.7	8.2	9.2
	Lymphocyte	4.1	4.6	4.9	4.6	4.7	4.6	4.5	5.1
	Neutrophil	3.6	2.6	2.9	3.4	3.2	3.0	2.9	3.0
	Eosinophil	0.44	0.71	0.37	0.81	0.92	0.76	0.64	0.83
0	Leukocyte	8.8	7.9	9.5	8.9	9.6	8.1	8.0	7.8
	Lymphocyte	5.2	4.5	5.5	5.1	5.0	4.7	4.6	4.3
	Neutrophil	2.8	2.6	3.0	3.2	3.5	2.6	2.7	2.5
	Eosinophil	0.62	0.82	0.81	0.52	0.94	0.86	0.55	0.58
24	Leukocyte	11.7*	11.1*	10.5	11.2	13.2*	15.4*	9.3	10.1
	Lymphocyte	4.7	4.7	4.9	5.3	5.6	4.2	4.6	4.8
	Neutrophil	6.2†	6.1†	4.8	5.3*	6.6*	10.9†	4.0	4.4†
	Eosinophil	0.51	0.15	0.49	0.37	0.65	0.14	0.60	0.78
48	Leukocyte	9.7	9.1	8.2	10.0	10.7	11.8*	8.0	10.0
	Lymphocyte	5.0	4.1	4.6	5.8	5.3	4.5	4.9	4.6
	Neutrophil	3.6	3.5	2.8	3.4	4.5	7.0*	2.2	4.4*
	Eosinophil	0.81	0.68	0.57	0.71	0.82	0.22	0.79	0.93
96	Leukocyte	8.2	7.0	6.9	8.1	8.8	12.0*	7.6	8.2
	Lymphocyte	4.8	4.2	3.7	4.6	4.6	4.7	4.1	4.2
	Neutrophil	2.8	2.2	2.4	2.9	3.0	4.2*	2.4	3.0
	Eosinophil	0.47	0.45	0.67	0.54	0.99	0.41	0.81	0.87
144	Leukocyte	7.2	7.5	6.7	7.7	8.8	9.1	7.3	8.0
	Lymphocyte	4.2	4.6	3.9	4.9	4.7	4.8	4.3	4.7
	Neutrophil	2.3	2.2	2.1	2.2	3.0	3.5	2.2	2.3
	Eosinophil	0.65	0.50	0.67	0.75	0.80	0.59	0.66	0.92

* P = 0.05; † P = 0.01.

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part, the enhanced potency of this steroid. Glenn *et al.*[4] reported that for analogues active as glucocorticoids, a fairly close correlation existed between increased biological activity and increased resistance to enzymatic attack in the liver.

The blood glucose response following the administration of the four synthetic compounds indicates that Δ' 9 α FF is the most potent and the most prolonged in this respect. Similar responses in blood glucose values were manifested by the administration of 100 mg. of Δ' F and 9 α FF, and by 25 mg. of Δ' 9 α FF, indicating that Δ' F and 9 α FF are about equal in their glyconeogenic properties, whereas Δ' 9 α FF is four times as potent as either of these.

Link *et al.*[7] found an average increase in blood glucose of 30 mg./100 ml. over a 24-hour period when 100 mg. of prednisolone was administered to ketotic dairy cattle. This increase is slightly greater than that found in the normal animal. The blood glucose-elevating properties of 9 α FF has been reported to be 15 to 20 times as great as hydrocortisone in the cow.[12]

The eosinopenic response to the administration of adrenal hormones and to stressors is well known. The decrease in circulating eosinophils following ACTH administration has been suggested as a possible method for evaluating adrenal cortical function in the cow[5] and dog.[9] In the cow, the mild eosinopenia but marked neutrocytosis reported herein following the administration of hydrocortisone analogues indicates that the neutrophilic response may be a more reliable indicator of adrenal function than decreased eosinophil numbers, although the compounds used in this study are not natural corticosteroids.

Some confusion exists on the effects and role of mineralocorticoids in cattle. Carlstrom[2] reported an increase in plasma potassium values following the administration of adrenal cortical extract or desoxycorticosterone to ketotic cows, whereas Shaw[12] found that the administration of 100 mg. of 9 α FF to ketotic cows depressed plasma potassium values as much as 50 per cent in 24 hours. The effects of adrenal cortical hormones on plasma potassium levels in normal cattle have been reported to be irregular.[2] In the present study, the failure to detect any changes in plasma sodium or potassium following the administration of synthetic corticoids does little to clarify the role of mineralocorticoids in cattle, and further study is needed.

SUMMARY

Prednisolone, 9 α fluorohydrocortisone, and 9 α fluoroprednisolone are powerful in their ability to induce an elevation of blood glucose in the normal lactating cow. Of these, 9 α fluoroprednisolone was approximately 4 times as potent in its glucogenic effects, when compared with prednisolone and 9 α fluorohydrocortisone. All compounds tested produced various degrees of leukocytosis and neutrocytosis. The greatest and most prolonged effects on the white cell picture were produced by 9 α fluoroprednisolone. No changes in plasma sodium or plasma potassium were detected following the administration of the synthetic corticoids tested.

References

[1] Bunim, J. J., Pechet, M. M., and Bollet, A. J.: Studies on Metacortandralone and Metacortandracin in Rheumatoid Arthritis. J. Am. M. A., *157*, (1955): 311.

[2] Carlstrom, B.: Deficiency Diseases, Particularly Acetonaemia in Cattle. Vet. Rec., *62*, (1952): 717.

[3] Collins, E. F., Forist, A. A., and Nadolski, E. B.: Δ'-Hydrocortisone and Hydrocortisone; Plasma 17-Hydroxycorticosteroid Concentrations in the Dog Following Oral and Intravenous Administration. Proc. Soc. Exptl. Biol. & Med., *93*, (1956): 369.

[4] Glenn, E. M., Stafford, R. O., Lyster, S. C., and Bowman, B. J.: Relation Between Biological Activity of Hydrocortisone and Analogues and Their Rates of Inactivation by Rat Liver Enzyme Systems. Endocrinology, *61*, (1957): 128.

[5] Hopwood, R. T., and Tibolla, B. J.: The Effect of Adrenocorticotrophic Hormone on the Circulating Eosinophil Level—A Possible Screening Test for Adrenal Gland Function in the Cow. Am. J. Vet. Res., *19*, (Oct., 1958): 833.

[6] Liddle, G. W., Pechet, M. M., and Bartter, F. C.: Enhancement of Biological Activities of Corticosteroids by Substitution of Halogen Atoms in 9 α Position. Science, *120*, (1954): 496.

[7] Link, R. P., Newton, D. I., and Huber, W. B.: The Use of Prednisolone in Bovine Ketosis. J.A.V.M.A., *130*, (1957): 137.

[8] Lyster, S. C., Barnes, L. E., Lund, G. H., Meinzinger, M. M., and Byrnes, W. W.: Adrenal Cortical Activities of 6-Methyl-Δ'-hydrocortisone. Proc. Soc. Exptl. Biol. & Med., *96*, (1957): 159.

[9] Martin, J. E., Skillen, R. G., and Deubler, M. J.: The Action of Adrenocorticotrophic Hormone on Circulating Eosinophils in Dogs—A Proposed Screening Method for Evaluating Adrenal Cortical Function. Am. J. Vet. Res., *15*, (Oct., 1954): 489.

[10] Nelson, H.: A Photometric Adaptation of the Somogi Method for the Determination of Glucose. J. Biol. Chem., *158*, (1944): 375.

[11] Perlman, P., and Tolksdorf, S.: Adrenocortical Activity of Meticorten and Meticortalone. Fed. Proc., *14*, (1955): 377.

[12] Shaw, J. C.: Ketosis in Dairy Cattle. A Review. J. Dai. Sci., *39*, (1956): 402.

[13] Stafford, R., Barnes, L., Bowman, B., and Meinzinger, M.: Glucocorticoid and Mineralocorticoid Activi-

ties of 9 α-Fluorohydrocortisone. Proc. Soc. Exptl. Biol. & Med., *89*, (1955): 371.

[14] Swingle, W. W., Brannick, L. J., Barlow, A. F., Baker, C., and LeBrie, S.: Life Maintenance and Mineralocorticoid Activity of Some New Derivative of Cortisone, Hydrocortisone, and Desoxycorticosterone. Endocrinology, *59*, (1956): 226.

[15] West, K. M.: Comparison of the Hyperglycemic Effects of Glucocorticoids in Human Beings. Diabetes, *6*, (1957): 175.

Two Projections into the Cranial Cavity Associated with Achondroplastic Dwarfism in Cattle

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THIS IS A REPORT of two projections into the cranial cavities of cattle and of the relationship of these projections to achondroplastic bovine dwarfism. This type of dwarfism was first described by Johnson *et al.*[10] A more complete description was presented by Gregory *et al.*,[6] who called attention to changes observed in the shape of the skull. Buchanan *et al.*[3] reported two types of modifications of the occipital condyles in dwarf cattle, both characterized by "crowding." Premature fusion of the spheno-occipital synchondrosis in dwarf cattle was reported by Julian *et al.*,[11] who discussed the possibility that altered growth of this region could be responsible for the changes observed in the skulls of dwarfs, and established achondroplasia as the dwarfing process. The diagnosis of the dwarfing process was later confirmed by Tyler *et al.*[15]

MATERIALS AND METHODS

The heads of 144 cattle necropsied for the study of bovine dwarfism were observed. All major beef breeds of cattle and crosses between these breeds were represented. The cattle were 1 day to 20 months old and included males, females, and a few steers. The animals were divided into groups according to presence or absence of achondroplasia. Criteria used included the values of three metacarpal indexes and the presence of fusion of the spheno-occipital synchondrosis.[11, 15, 16] Animals in group 1 did not manifest any of the four objective characteristics which were used to diagnose achondroplasia. Many were descended from ancestors that carried dwarfism, and some were proven dwarf carriers. Many of these animals were subnormal in body weight and measurements, and may prove to be achondroplastic when new objective criteria are developed.

These animals were a heterogeneous group. In this report, group 1 is referred to as the "nonachondroplastic" group. Animals in group 2 were achondroplastic by all four of the criteria. Animals in groups 3 and 4 showed localized achondroplasia and partial achondroplasia, respectively (achondroplastic by one or more criteria, but nonachondroplastic by the other criteria). In this study, groups 3 and 4 were combined to simplify the presentation of the data. When combined, these two groups constituted only 27 animals, or 19 per cent of the total examined. Of the 144 animals, 51 per cent (73 animals) were classified as group 1. Group 2 contained 30 per cent (44 animals) of the animals.

The heads were sectioned sagittally, the brains were removed, and the presence and severity of the projections were observed. Projections were graded as slight, moderate, or severe. Criteria used in this classification included both the total length and the relative mass of the projection. Endocranial casts, representing both normal and pathological types, were made with white latex diluted 25 per cent with tap water.

More than 100 heads were observed at a local abattoir to determine the incidence of projections in the slaughter population. The observations were made on several days, and the animals included steers, heifers, and veal calves. All of these animals were presumed to be nonachondroplastic.

RESULTS

The position of the two projections (fig. 1) and a normal sagittally sectioned skull (fig. 2) are shown. In affected cattle, the wing of the orbitosphenoid (wos), ala minor, projects medially and dorsally into the cranial cavity. This projection may extend into the rhinal fissure of the brain, or it may press on the insular area of the

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Dr. Evans was on sabbatical leave from the Department of Anatomy, New York State Veterinary College, Ithaca.

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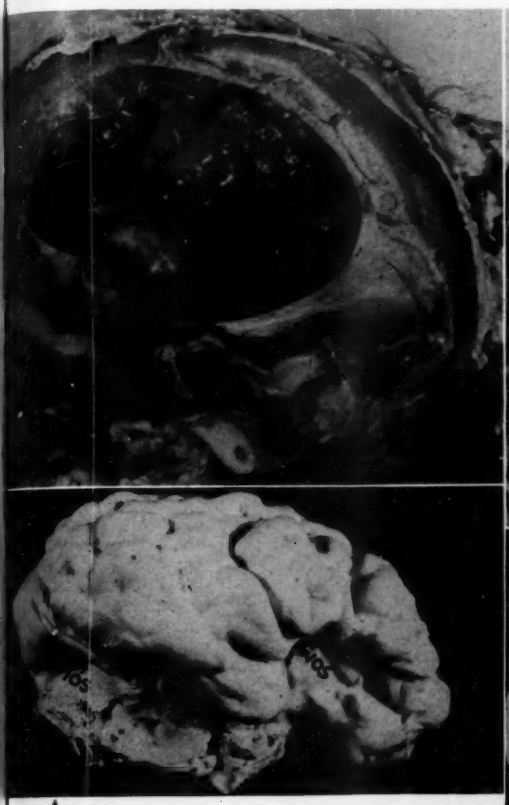


Fig. 1—Sagittally sectioned bovine skull and endocranial cast showing severe projections of the wing of the orbitosphenoid (WOS) and the posterior intraoccipital synchondrosis (PIOS).

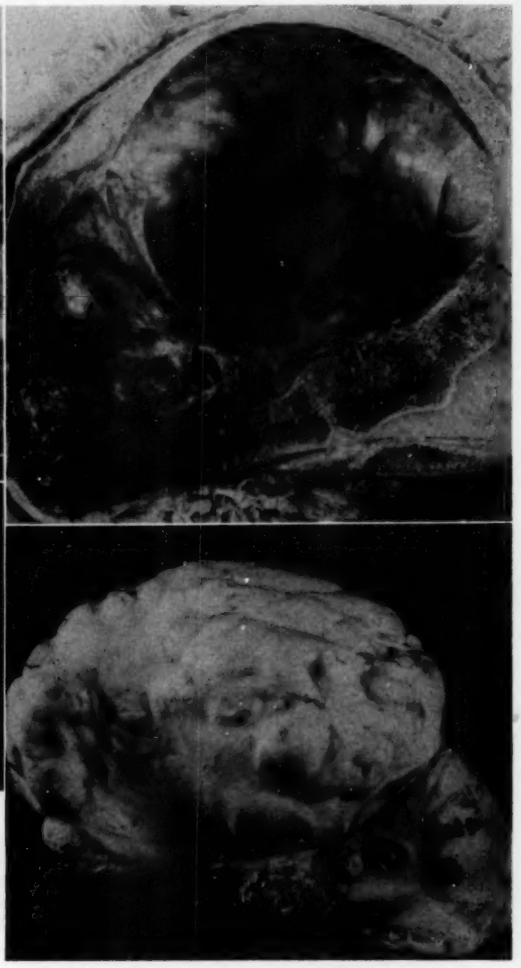
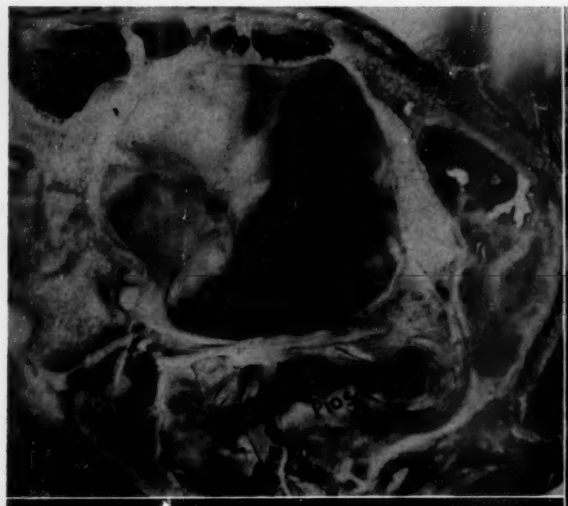


Fig. 2—A normal sagittally sectioned skull and endocranial cast (right).

cerebral cortex. The wos projection includes both the cartilaginous extremity and a portion of the osseous ala. This lesion appears less severe in older animals. The posterior intraoccipital synchondrosis (PIOS), the synchondrosis between the exoccipital and the supraoccipital, projects medially and anteriorly into the posterior portion of the cranial cavity (fig. 1). This projection presses into the cerebellar hemisphere. The cerebellar cortex may be displaced, exposing the corpus medullare. The internal surface of the cranium of all involved animals presented sharp, bony ridges which entered the sulci. Either projection occurred individually (fig. 3, 4), or both projections occurred in the same skull (fig. 1).

The occurrence, incidence, and severity of the projections are presented (table 1). The incidence of wos projections in the nonachondroplastic group 1 was 37 per cent. In group 2, wos projections were observed in 77 per cent of the animals. In combined groups 3 and 4, the incidence was 48 per cent. In group 2, PIOS projections were observed in 100 per cent of the animals, but in group 1 the incidence was only 23 per cent. Combined groups 3 and 4 had an incidence of 52 per cent.

The incidence of only the moderate and severe wos projections was 8 per cent in



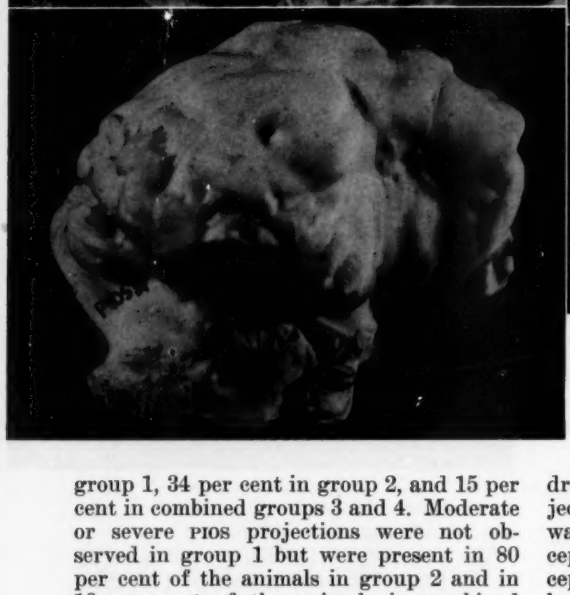


Fig. 3 (left)—Sagittally sectioned bovine skull and endocranial cast showing severe projection of only posterior intraoccipital synchondrosis (PIOS).
Fig. 4 (above)—Sagittally sectioned skull and endocranial cast showing severe projection of only the wing of the orbitosphenoid (WOS).

group 1, 34 per cent in group 2, and 15 per cent in combined groups 3 and 4. Moderate or severe PIOS projections were not observed in group 1 but were present in 80 per cent of the animals in group 2 and in 19 per cent of the animals in combined groups 3 and 4. Thus, both the incidence and the severity of these projections were greatest in the achondroplastic cattle in group 2, intermediate in the locally and partially achondroplastic animals of groups 3 and 4, and lowest in the non-achondroplastic cattle in group 1.

Included in this report were 3 Hereford calves which had extreme hydrocephalus. Using the criteria described previously, these animals were classified as nonachondroplastic. None of the 3 calves had projections in either area. This observation was especially interesting, as mild hydrocephalus has been observed in the brachycephalic dwarf.[5] Additional animals must be examined before the relationship of these projections to the extreme hydrocephalic condition can be established.

No projections were found in the apparently normal cattle observed at a local abattoir.

DISCUSSION

The observations indicate a correlation between the projections and achondroplastic dwarfism. This correlation permits an explanation of the development of the

lesions. Julian *et al.*[11] and Tyler *et al.*[15] established hypoplastic achondroplasia as the process responsible for the brachycephalic or "short-headed" Hereford dwarf. Gregory and Carroll[7] demonstrated that the same gene is responsible for this type of dwarfism in the major beef breeds of cattle. It appears logical to assume that the dwarfing process is identical in phenotypically similar dwarfs in any of these breeds.

Observations on bovine embryos, prepared according to the method described by Evans,[4] have demonstrated that the orbitosphenoid develops medially to the orbital portion of the frontal bone. These observations were confirmed by gross inspection of the skulls of normal young calves. The sphenofrontal suture is characterized by an overlap rather than an appositional union of the free edges of the growing bones. In animals with wos projections,

TABLE 1—Occurrence, Incidence, and Severity of Projections in Dwarf Cattle

Group	Normal		Slight		Moderate		Severe	
	No.	(%)	No.	(%)	No.	(%)	No.	(%)
WING OF THE ORBITOSPHENOID PROJECTIONS								
Group 1 (Nonachondroplastic)	46	63.0	21	28.8	5	6.8	1	1.4
Group 2 (Achondroplastic)	10	22.7	19	43.2	7	15.9	8	18.2
Groups 3 and 4 (Local and partial achondroplastic)	14	51.8	9	33.3	2	7.4	2	7.4
POSTERIOR INTRAOCCIPITAL SYNCHONDROSIS PROJECTIONS								
Group 1 (Nonachondroplastic)	56	76.7	17	23.3	0	0.0	0	0.0
Group 2 (Achondroplastic)	0	0.0	9	20.4	24	54.5	11	25.0
Groups 3 and 4 (Local and partial achondroplastic)	13	48.1	9	33.3	3	11.1	2	7.4

Achondroplasia only influences the interstitial growth of cartilage.[17] Therefore, this disease primarily influences bones formed by endochondral ossification but only secondarily influences bones resulting from intramembranous ossification. Crew[3] observed a reduction in length of the endochondral bones which form the base of the skull in the type of achondroplastic bovine dwarf which he described. In achondroplastic dwarfs of other species, Benda[1] and Stein *et al.*[14] reported that the endochondral bones of the cranium of achondroplastic dwarfs were subnormal in size. Sawin and Crary[13] used quantitative anatomic techniques to demonstrate the localized reduction in length and width of the endochondral skull bones of the achondroplastic rabbit. They reported that the Dachs gene appears to cause a relatively greater reduction in the width than in the length of these bones. Preliminary results of a similar study of the bovine dwarf also indicate that these bones are reduced in size. The "pinched" condyles described by Buchanan *et al.*[2] may also be considered evidence of a reduction of the width of the occipital bone.

this suture is apparently imperfectly formed because of the arrested development of the sphenoid and the relatively normal growth of the frontal bones. The suture appears to be less extensive in these animals and only involves the base of the orbitosphenoid, allowing the major portion of the ala to project into the cranial cavity.

The concept that the dwarfing process directly influences endochondral bone but only secondarily influences intramembranous bone also provides the basis for an explanation of the projections of the posterior intraoccipital synchondrosis. Differential growth of bones formed by these two types of ossification produces abnormal stresses on the connections between the cranial base and vault, resulting in the invagination of this synchondrosis and the adjoining portions of the occipital bone.

The relationship of these projections to the muscular incoordination frequently observed in dwarf cattle is not clear. All of the achondroplastic cattle in group 2 had PIOS projections which could possibly be responsible for some of the observed muscular incoordination. Severe nervous signs were observed in a number of the 144 ani-

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mals examined. One of these animals was depressed and later showed nystagmus and opisthotonos. A few days later, this animal died of an acute septicemia. It was found to have a severe projection of the wos and a normal PIOS (fig. 3). Other animals with nervous signs showed exaggerated cord reflexes and an incoordination which became less pronounced with increasing age. Some calves were unable to stand. Many of these animals had moderate or severe projections in one or both areas. The neuropathy resulting from these projections is being studied, as is the neuropathy due to the hydrocephalus associated with bovine dwarfism and to the extreme hydrocephalic condition.

The value of these projections as aids to the diagnosis of achondroplastic dwarfism is not entirely clear. The evidence strongly indicates a correlation between these projections and this type of dwarfism. The confusion arises from the presence of projections in the nonachondroplastic cattle in group 1. It should be recalled that most cattle of this group were descended from dwarf carrier stock, subnormal in size, and some were proven dwarf carriers. Thus, part of this confusion is due to the lack of an adequate control or normal population. Objective standards must be developed to define the normal animals and each specific type of mutant. The absence of these projections in apparently normal slaughter cattle indicates that they may represent a more adequate control population than the nonachondroplastic cattle in group 1. These observations also support the concept that bovine dwarfism is a complex [12] and are in agreement with genetic data which also indicate a complex.[8,9]

The PIOS projection may have some diagnostic value in animals too young to exhibit fusion of the spheno-occipital synchondrosis [11] and whose metacarpal indixes are not diagnostic.[16] The WOS characteristic may prove to be of value in the differential diagnosis of different components of the dwarf complex.

SUMMARY

Two projections into the cranial cavity of cattle were reported. The occurrence and the degree of severity of these projections were observed in 144 animals necropsied for the study of bovine dwarfism. The incidence and severity of projections in the achondroplastic animals of group 2 was greater than that of the nonachondroplastic control animals, indicating a correlation between these projections and dwarfism.

No projections were observed in a sample of over 100 animals slaughtered at a local abattoir. An explanation of the development of these projections which involves arrested development and differential growth rates due to achondroplasia was proposed. The presence of nervous signs in some animals with these projections was noticed. The possible value of these projections as aids to the diagnosis of achondroplastic dwarfism and the differentiation of members of the dwarf complex was discussed.

References

[1] Benda, C. E.: Mongolism and Cretinism. 2nd ed. Grune and Stratton, New York, N.Y. 1949.

[2] Buchanan, M. L., Bolin, F. M., Burham, J. J., and Eveleth, D. F.: Some Skeletal Changes Associated with Dwarfism in Cattle. North Dakota Exper. Station Bull. 403 (1956): 1–6.

[3] Crew, F. A. E.: The Significance of an Achondroplasia-like Condition Met Within Cattle. Proc. Roy. Soc. London s.B, (1923): 228–255.

[4] Evans, H. E.: Clearing and Staining Small Vertebrates, in Toto, for Demonstrating Ossification. Turtox News, *26*, (1948): 1–6.

[5] Fransen, J. M., and Andrews, F. N.: Cerebrospinal Fluid Pressure in Dwarf and Normal Cattle. Am. J. Vet. Res., *19*, (April, 1958): 336–337.

[6] Gregory, P. W., Rollins, W. C., Pattengale, P. S., and Carroll, F. D.: A Phenotypic Expression of Homozygous Dwarfism in Beef Cattle. J. Anim. Sci., *10*, (1951): 922–933.

[7] Gregory, P. W., and Carroll, F. D.: Evidence for the Same Dwarf Gene in Hereford, Aberdeen Angus, and Certain Other Breeds of Cattle. J. Hered., *47*, (1956): 107–111.

[8] Gregory, P. W.: Phenotypic Forms and the Genetic Relationships of the Bovine Dwarf Complex. Abstr. in J. Anim. Sci., *15*, (1956): 1207.

[9] Gregory, P. W., Julian, L. M., and Tyler, W. S.: The Validity of Dwarf-Carrier Cows as Testors for the Major Dwarf Conditioning Gene. Abstr. in J. Anim. Sci., *16*, (1957): 1028.

[10] Johnson, L. E., Harshfield, G. S., and McCone, W.: Dwarfism, a Hereditary Defect in Beef Cattle. J. Hered., *41*, (1950): 177–181.

[11] Julian, L. M., Tyler, W. S., Hage, T. J., and Gregory, P. W.: Premature Closure of the Spheno-Occipital Synchondrosis in the Horned Hereford Dwarf of the "Short-Headed" Type. Am. J. Anat., *100*, (1957): 269–288.

[12] Julian, L. M., Tyler, W. S., and Gregory, P. W.: The Current Status of Bovine Dwarfism. J.A.V.M.A., *135*, (July 15, 1959): 104–109.

[13] Sawin, P. B., and Crary, D. D.: Morphogenetic Studies of the Rabbit. XVII. Disproportionate Adult Size Induced by the Da Gene. Genetics, *43*, (1957): 72–91.

[14] Stein, I., Stein, R. O., and Beller, M. L.: Living Bone in Health and Disease. J. P. Lippincott Co., Philadelphia, 1955.

[15] Tyler, W. S., Julian, L. M., and Gregory, P. W.: The Nature of the Process Responsible for the Short-

Headed Hereford Dwarf as Revealed by Gross Examination of the Appendicular Skeleton. Am. J. Anat., *101*, (1957): 477–496.

[16] Tyler, W. S., Julian, L. M., and Gregory, P. W.; University of California, Davis: Unpublished data, 1959.

[17] Weinmann, J. J., and Sicher, H.: Bone and Bones. Fundamentals of Bone Biology. 2nd ed. C. V. Mosby Co., St. Louis, Mo. 1955.

Treatment of Experimental Bovine Coccidiosis with Multiple Small Doses and Single Large Doses of Sulfamethazine and Sulfabromomethazine

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COCCIDIOSIS IN CATTLE may be successfully controlled with sulfaguanidine,[1] sulfamethazine,[3, 4, 6] sulfamerazine[5] and, somewhat less successfully, with sulfaquinoxaline.[3, 5] Timing of treatment with respect to stage of the disease is of critical importance. It has been shown that when calves experimentally infected with *Eimeria bovis* were given sulfonamides 13, 14, 15, and 16 days after inoculation, little or no injury occurred; treatment at other stages of the disease was much less effective.[5] Preliminary experiments indicated that treatment might be simplified either by reducing dosage to a single administration or by a form of continuous low-level treatment.

This experiment was conducted to determine the value of these methods of treatment with sulfabromomethazine, a drug reported to persist at fairly high levels in the blood stream for relatively long periods,[8] and with sulfamethazine.

MATERIALS AND METHODS

After completion of three preliminary experiments, five experiments involving 49 calves were carried out from August, 1957, to May, 1958. All calves were Holstein-Friesian males, obtained when 1 to 4 days old and housed in individual outdoor pens, similar to those described by Davis.[2] Each calf was fed approximately 8 lb. of whole milk per day for the first four or five weeks and, thereafter, about 10 lb. of nonfat milk reconstituted from powder. Alfalfa hay and a grain mixture were provided as a supplement to the milk after the second week. Vitamin A was given daily.

Daily collections of fecal samples were begun when the calves were 2 to 3 weeks old. A slightly modified McMaster technique[9] was used in estimating the number of oocysts per unit volume of feces. The calves were weighed at weekly intervals.

In each experiment, the calves were allotted randomly to three or four groups, usually with 4 calves in each group. When 1 to 3 weeks old, the calves were inoculated with sporulated oocysts, chiefly *E. bovis*, by means of a nippled flask. In each experiment, each group of calves was treated differently with sulfabromomethazine or sulfamethazine administered in gelatin capsules, except for one group which served as a control. Each experiment was continued for about 30 days.

EXPERIMENTAL PROCEDURES AND RESULTS

Preliminary Experiments.—In one preliminary experiment with 16 calves, each calf was inoculated with about 650,000 oocysts of *E. bovis* and sulfabromomethazine was administered once 13 days after inoculation or twice 13 and 15 days after inoculation, at the rate of 1.5 gr. per pound of body weight. Control of coccidiosis was as good as in treatment at the same rate with a mixture of equal parts of powdered sulfamerazine and sulfamethazine* given 13, 14, 15, and 16 days after inoculation.

In two other preliminary experiments, the results were not clearly defined. In the first of these two experiments, the calves were given either a single dose of sulfabromomethazine or of sulfamethazine (1.5 gr./lb.) or they were treated with 13 small doses of sulfabromomethazine (0.15 gr./lb.) on alternate days. Although natural infections were more prevalent than usual, even the untreated calves did not develop clinical coccidiosis. In the second experiment, with similar treatments, the same calves were used with the addition of new control calves, and resistance to reinfection was evident. Therefore, this experiment was repeated.

Experiment 1.—The 4 calves in each

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* Merameth, a mixture produced by Merck Sharp and Dohme Laboratories, Division of Merck and Co., Inc., West Point, Pa.

of four groups were each inoculated with approximately 500,000 oocysts. Those in one group were each treated with sulfabromomethazine (0.15 gr./lb.) in each of 13 treatments on alternate days over a 25-day period, beginning three days prior to inoculation. Those in a second group were given sulfabromomethazine (1.5 gr./lb.) 13 days after inoculation. Those in a third group were given sulfamethazine at the same dosage rate and time as the second group. Calves in the fourth (control) group were not treated.

Three of the 4 control calves developed severe coccidiosis, resulting in one death 27 days after inoculation (table 1). In contrast, none of the treated calves showed signs of coccidiosis other than diarrhea of brief duration, and none discharged more than a relatively few oocysts. There was no significant difference between any animals of the treated groups, either in numbers of oocysts discharged or in signs of coccidiosis.

Experiment 2.—The 4 calves in each of four groups were each inoculated with approximately 1 million oocysts. The calves in one group were given sulfabromomethazine (0.15 gr./lb.) in each of eight treatments on alternate days from ten to 24 days after inoculation. Those in a second group were given sulfabromomethazine (1.5 gr./lb.) 14 days after inoculation, and those in a third group were similarly treated with sulfamethazine. The 4 control calves were given no treatment.

Three of the 4 control calves developed severe coccidiosis, but none died during the experiment (table 2). The calves given the low-level treatment with sulfabromomethazine and those treated with a single large dose of sulfamethazine exhibited only

TABLE 1—Results of Treatment with 13 Small Doses of Sulfabromomethazine and with Single Large Doses of This Drug and of Sulfamethazine in Calves Inoculated with Approximately 500,000 Eimeria Bovis Oocysts (Experiment 1)

Group and treatment	Calf No.	Oocyst discharge*		Clinical signs		Weight gained (30 days)
		Peak-days after inoc.	Duration (days)	Diarrhea (days)	Bloody feces (days)	
1	298	16–20	7	5	0	30
Low-level	299	104–22	9	3	0	20
treatment†	300	9–17	3	0	0	26
	276	5–18	15	3	0	27
Mean		33.5–19.25	8.5	2.75	0	27.75
2	176	8–26	12	0	0	16
Single dose	177	27–28	5	0	0	19
sulfabromo-	178	10–17	1	1	0	14
methazine **	364	10–17	16	0	0	15
Mean		13.75–22.0	8.5	0.25	0	16.0
3	179	10–25	13	1	0	32
Single dose	353	0–0	0	0	0	.... ***
sulfamethazine **	296	49–27	14	1	0	27
	297	1–26	3	0	0	16
Mean		15.0–19.5	7.5	0.5	0	18.75
4	351	1,325–17	16	13	10	—8
No treatment	352	980–24	11	8	8	....‡
	354	348–20	16	11	4	5
	355	21–21	10	0	0	31
Mean		668.5–20.5	13.25	8.0	5.5	9.33

ANALYSIS OF VARIANCE

Source	Degrees of freedom	Mean squares			
		Peak oocyst count	Duration of oocyst discharge	Diarrhea (days)	Bloody feces (days)
Treatment	3	419,906.30‖	26.73	51.75‖	30.25§
4 vs. 1, 2, 3	1	1,258,740.18§	77.52	140.08§	90.75§
1 vs. 2	1	780.13	0.0	12.50	0
1 vs. 2, 3	1	975.37	0.67	15.04	0
2 vs. 3	1	12.13	2.00	0.13	0
Error term	12	88,210.71	32.65	9.375	5.75

* Peak = No. of oocysts per 1/360 cc. (0.003 cc.) of fecal sample; duration = days with discharge of 360 or more oocysts per cubic centimeter of fecal sample. † Treatment consisted of 0.15 gr/lb. of body weight per treatment on alternate days from three days before inoculation until 21 days after. ** Treatment consisted of 1.5 gr./lb. of body weight given 13 days after inoculation. *** Died 27 days after inoculation, cause unknown. ‡ Died of coccidiosis 25 days after inoculation. ‖ Significant at the 0.01 level. § Significant at the 0.05 level.

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mild or no signs of coccidiosis, and the level of oocyst discharge was relatively low. The calves treated with a single dose of sulfabromomethazine developed coccidiosis that was about as severe as that developed by control animals, but for a shorter period, and fewer oocysts were discharged. However, one of these calves died 21 days after inoculation, apparently of coccidiosis.

Experiment 3.—The 4 calves in each of three groups were inoculated with approximately 1 million oocysts. Those in the first group were given sulfabromomethazine (0.15 gr./lb.) in each of five treatments on alternate days from ten to 18 days after inoculation. Those in a second group were similarly treated with sulfamethazine. Calves in the control group were not treated. One calf in the first group and 1 control calf died before the period at which the signs of coccidiosis were expected to appear (table 3).

All of the 3 surviving control calves developed severe coccidiosis; one of these died on the twentieth day and another on the twenty-fifth day after inoculation, apparently as a result of the infection. The treated calves showed no signs or only mild signs of coccidiosis, except for 1 of the 3 surviving calves which had been treated with sulfabromomethazine. This calf had bloody diarrhea for two days; the level of oocyst discharge was low and the gain in weight relatively good. None of the treated calves discharged large numbers of oocysts.

Experiment 4.—In this experiment, the calves which survived experiment 3 were given a challenge inoculation to evaluate their resistance to reinfection. Included in the experiment were 3 calves which had been given five low-level treatments with sulfabromomethazine on alternate days from 10 to 18 days after inoculation, 4 calves which had been similarly treated

TABLE 2—Results of Treatment with Eight Small Doses of Sulfabromomethazine and with a Single Large Dose of this Drug and of Sulfamethazine in Calves Inoculated with Approximately 1,000,000 Eimeria Bovis Oocysts (Experiment 2)

Group and treatment	Calf No.	Oocyst discharge*		Clinical signs		Weight gained (30 days)
		Peak-days after inoc.	Duration (days)	Diarrhea (days)	Bloody feces (days)	
1	376	25–19	3	2	0	25
Low-level	377	19–19	6	5	0	23
treatment	393	6–19	1	0	0	24
	379	5–19	2	1	0	20
Mean		13.75–19.0	3.0	2.0	0	23.0
2	380	180–24	13	1	0	20
Single dose	381	154–19	12	14	9	−17
sulfabromo-	382	162–18	6	5	4	....***
methazine **	383	12–20	8	8	2	5
Mean		127.25–20.25	9.75	7.0	3.75	2.66
3	384	64–19	6	5	0	23
Single dose	385	56–19	3	1	0	22
sulfamethazine **	386	44–19	7	0	0	18
	389	86–19	5	0	0	11
Mean		62.5–19.0	5.25	1.5	0	18.5
4	387	206–22	13	10	3	8
Control	390	210–22	14	11	0	5
	391	1,188–23	16	13	13	4
	392	702–22	9	11	6	5
Mean		576.5–22.75	13.0	11.25	5.5	5.5

ANALYSIS OF VARIANCE

Source	Degrees of freedom	Mean squares			
		Peak oocyst count	Duration of oocyst discharge	Diarrhea (days)	Bloody feces (days)
Treatment	3	267,432.06‡	80.50§	84.73§	38.98
4 vs. 1, 2, 3	1	776,479.69§	147.00§	180.19§	54.19
1 vs. 2	1	25,651.13	91.13	50.00‡	24.07
2 vs. 3	1	8,320.50	40.50‡	59.25‡	24.07
Error term	12	56,707.73	6.79	10.40	9.38

* Peak = No. of oocysts per 1/360 cc. (0.003 cc.) of fecal sample; duration = days with discharge of 360 or more oocysts per cubic centimeter of fecal sample. † Treatment consisted of 0.15 gr. of sulfabromomethazine per pound of body weight per treatment on alternate days from ten to 24 days after inoculation. ** Treatment consisted of 1.5 gr. per pound of body weight given 14 days after inoculation. *** Died of coccidiosis 21 days after inoculation. ‡ Significant at the 0.05 level. § Significant at the 0.01 level.

TABLE 3—Results of Treatment with Five Small Doses of Sulfabromomethazine and of Sulfamethazine in Calves Inoculated with Approximately 1,000,000 Eimeria Bovis Oocysts (Experiment 3)

Group and treatment	Calf No.	Oocyst discharge*		Clinical signs		Weight gained (30 days)
		Peak-days after inoc.	Duration (days)	Diarrhea (days)	Bloody feces (days)	
1	400	20–20	9	0	0	32
Sulfabromo-	396	.....	....	....	..	....**
methazine†	416	10–19	3	6	2	25
	395	132–21	3	0	0	33
Mean		54.0–20.0	5.0	2.0	0.66	30.0
2	397	1–19	4	1	0	23
Sulfamethazine†	419	5–19	1	3	0	31
	420	84–20	5	0	0	41
	421	85–20	5	2	0	37
Mean		58.33–19.5	3.75	1.5	0.0	33.0
3	422	2,800–21	9	9	6	....***
Control	513	3,000–20	6	4	2	....‡
	425	1,200–21	13	13	4	1
	514	.....	....	....	..	....‖
Mean		2,333.0–20.66	9.33	8.66	4.0	1.0

ANALYSIS OF VARIANCE

Source	Degrees of freedom	Mean squares			
		Peak oocyst count	Duration of oocyst discharge	Diarrhea (days)	Bloody feces (days)
Treatment	2	5,483,287.18§	28.09	52.47	14.65§
3 vs. 1, 2	1	10,966,400.27§	53.50¶	101.50§	28.97§
1 vs. 2	1	180.11	2.68	3.43	0.76
Error term	7	280,356.25	8.49	9.53	1.52

* Peak = No. of oocysts per 1/360 cc. (0.003 cc.) of fecal sample; duration = days with discharge of 360 or more oocysts per cubic centimeter of fecal sample. † Treatment consisted of 0.15 gr. of drug per pound of body weight per treatment on alternate days from ten to 18 days after inoculation. ** Died 16 days after inoculation, cause unknown. *** Died of coccidiosis 25 days after inoculation. ‡ Died of coccidiosis 20 days after inoculation. ‖ Died ten days after inocualtion, cause unknown. § Significant at the 0.01 level. ¶ Significant at the 0.05 level.

TABLE 4—Results of an Inoculation with 3,000,000 Eimeria Bovis Oocysts of Calves Previously Inoculated and Treated with Five Small Doses of Sulfonamides (Experiment 4)

Group and treatment	Calf No.	Oocyst discharge*		Clinical signs		Weight gained (30 days)
		Peak-days after inoc.	Duration (days)	Diarrhea (days)	Bloody feces (days)	
1. Previously	400	2–25	3	1	0	47
treated with	416	4–22	5	0	0	41
sulfabromomethazine†	395	6–29	8	8	0	43
Mean		4.0–25.3	5.3	3.0	0.0	43.6
2	397	0–0	0	2	0	48
Previously	419	12–20	11	3	0	42
treated with	420	6–25	1	0	0	57
sulfamethazine†	421	1–21	2	0	0	57
Mean		4.75–22.0	3.5	1.25	0.0	51.0
3. Previously	32	1,446–21	6	5	3	....**
uninoculated	33	438–18	2	2	2	....***
(control)	35	1,200–20	7	5	4	....‡
Mean		1,028–19.6	5.0	4.0	3.66	.....

ANALYSIS OF VARIANCE

Source	Degrees of freedom	Mean squares			
		Peak oocyst count	Duration of oocyst discharge	Diarrhea (days)	Bloody feces (days)
Treatment	2	1,100,083.88§	3.42	6.83	9.45
1 vs. 2	1	0.97	5.76	5.25	0.0
3 vs. 1, 2	1	2,200,166.78§	16.07	3.50	0.0
Error	7	78,929.54	14.81	7.25	0.29

* Peak = No. of oocysts per 1/360 cc. (0.003 cc.) of fecal sample; duration = days with discharge of 360 or more oocysts per cubic centimeter of fecal sample. † Treated on alternate days at the rate of 0.15 gr. per pound of body weight from 12 to 18 days after the previous inoculation. ** Died of coccidiosis 21 days after inoculation. *** Died of coccidiosis 18 days after inoculation. ‡ Died of coccidiosis 22 days after inoculation. § Significant at the 0.01 level.

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with sulfamethazine, and 3 previously uninoculated control calves. Each of the calves was given approximately 3 million oocysts 36 days after the previous inoculation. At the time of this inoculation, the 3 control calves were about 3 weeks old, whereas the other calves were about 7 weeks old.

All of the control calves developed severe coccidiosis, resulting in their deaths, 18, 21, and 22 days after inoculation (table 4). None of the previously inoculated and treated calves developed any more than mild signs of coccidiosis, and none discharged more than relatively small numbers of oocysts. The results clearly indicate that the treatment had not appreciably interfered with the development of immunity.

Experiment 5.—The 6 calves used in two groups in this experiment were each inoculated with approximately 1 million oocysts. The 3 calves in one group were each given sulfamethazine (0.15 gr./lb.) 12 and 14 days after the inoculation. The other 3 calves were not treated.

One of the control calves died before the period when signs of coccidiosis might be expected. Both of the surviving controls developed severe coccidiosis and 1 of these died 22 days after inoculation, apparently as a result of the infection. None of the treated calves showed any signs of coccidiosis and none discharged more than a few oocysts.

DISCUSSION

The results of this study show that experimental coccidiosis caused by *E. bovis* can be controlled with lower dosage levels and fewer administrations of sulfonamides than had been previously demonstrated. In experiments 1 and 2, coccidiosis was successfully treated with a single administration of sulfamethazine at 1.5 gr. per pound of body weight 13 or 14 days after inoculation and, in experiment 1, with a similar single dose of sulfabromomethazine 13 days after inoculation.

It is difficult to explain the failure of treatment with a single administration of sulfabromomethazine 14 days after inoculation in experiment 2. Both the solubility of sulfabromomethazine and the blood level 12 hours after oral administration were lower than with sulfamethazine.[8] Either or both of these characteristics may be responsible for the difference in results obtained with the two drugs when administered 14 days after inoculation.

Coccidiosis was also controlled, in experiments 2 and 3, by administration of either of these drugs on alternate days at the rate of 0.15 gr. per pound of body weight per treatment from 10 to 18 or 24 days after inoculation. In experiment 5, involving fewer calves than in the other experiments, treatment with sulfamethazine at the rate of 0.15 gr. per pound of body weight 12 and 14 days after inoculation apparently resulted in suppression of coccidiosis. These

TABLE 5—Results of Treatment with Two Small Doses of Sulfamethazine 12 and 14 Days After Inoculation with 1,000,000 Eimeria Bovis Oocysts (Experiment 5)

Group and treatment	Calf No.	Oocyst discharge*		Clinical signs		Weight gained (30 days)
		Peak-days after inoc.	Duration (days)	Diarrhea (days)	Bloody feces (days)	
1	39	12–19	2	0	0	20
Treated†	129	16–23	7	0	0	23
	142	9–19	2	0	0	23
Mean		12.3–20.3	3.66	0.0	0.0	22.0
2	38	150–19	6	9	2	29
Untreated (control)	146	1,062–20	7	6	5	....**
Mean		606.0–19.5	6.5	7.5	3.5	.....

ANALYSIS OF VARIANCE

Source	Degrees of freedom	Mean squares			
		Peak oocyst count	Duration of oocyst discharge	Diarrhea (days)	Bloody feces (days)
Treatment	1	422,982.13	9.63	67.5‡	14.7
Error	3	138,632.22	5.72	1.5	1.5

* Peak = No. of oocysts per 1/360 cc. (0.003 cc.) of fecal sample; duration = days with discharge of 360 or more oocysts per cubic centimeter of fecal sample. † Treated with sulfamethazine at the rate of 0.15 gr. per pound of body weight per treatment. ** Died of coccidiosis 22 days after inoculation. ‡ Significant at the 0.01 level.

results indicate that relatively low dosage levels of sulfonamides are sufficient to control infections with *E. bovis*, and that the drugs are probably most effective during a relatively short period in the life cycle of the parasite.

The results in experiment 4 show that low-level treatment with these sulfonamides, over a period of eight days during an infection with *E. bovis*, had no appreciable influence on development of immunity. This agrees with the previous finding [7] that immunity develops in spite of treatment with equal parts of sulfamerazine and sulfamethazine 13, 14, and 15 days after inoculation with *E. bovis*.

One of the most serious difficulties in controlling naturally acquired coccidiosis is that the presence of the disease is usually not recognized until after completion of the stage or stages most susceptible to treatment. Low-level administration of sulfonamides might provide ways of solving this problem. Continuous low-level feeding of drugs, as is done with poultry, might be practicable in special situations in which coccidiosis frequently occurs. Also, low-level treatment with sulfonamides could be started in all the members of a group of animals at the time coccidiosis is first diagnosed in one or more individuals in the groups, and could be continued for as long as necessary to provide control.

Further investigation is needed to determine whether these findings might also be applicable to coccidiosis caused by *Eimeria zurnii*.

Summary

Five experiments were conducted in which sulfamethazine or sulfabromomethazine was given, either in one or two large doses or in multiple small doses on alternate days, to 49 calves experimentally infected with *Eimeria bovis*.

A single treatment with sulfamethazine or sulfabromomethazine at the rate of 1.5 gr. per pound of body weight 13 days after inoculation effectively controlled coccidiosis, as did administration of either of these drugs on alternate days at the rate of 0.15 gr. per pound of body weight for as short a period as 10 to 18 days after inoculation.

Such low-level treatment did not appreciably interfere with development of immunity.

In one experiment, treatment with sulfamethazine at the low level (0.15 gr./lb.) 12 and 14 days after inoculation suppressed coccidiosis.

References

[1] Boughton, D. C.: Sulfaguanidine Therapy in Experimental Bovine Coccidiosis. Am. J. Vet. Res., *4*, (Jan., 1943): 66–72.

[2] Davis, L. R.: Portable Pens for Raising Healthy Dairy Calves. Mimeo., U.S.D.A., Washington, D.C., Jan. 1949, revised Sept. 1949.

[3] Davis, L. R., and Bowman, G. W.: Coccidiosis in Cattle. Proc. U.S. Livestock San. A., (1951): 39–50.

[4] Davis, L. R., and Bowman, G. W.: The Use of Sulfamethazine in Experimental Coccidiosis of Dairy Calves. Cornell Vet., *44*, (1954): 71–79.

[5] Hammond, D. M., Shupe, J. L., Johnson, A. E., Fitzgerald, P. R., and Thorne, J. L.: Sulfaquinoxaline and Sulfamerazine in the Treatment of Experimental Infections with *Eimeria Bovis* in Calves. Am. J. Vet. Res., *17*, (July, 1956): 463–470.

[6] Horton-Smith, C.: Coccidiosis in Domestic Mammals. Vet. Rec., *70*, (1958): 256–262.

[7] Senger, C. M., Hammond, D. M., Thorne, J. L., Johnson, A. E., and Wells, G. M.: Resistance of Calves to Reinfection with *Eimeria Bovis*. J. Protozool., *6*, (1959): 51–58.

[8] Stowe, C. M., Aronson, A. L., and Johnson, K.: The Pharmacology of Sulfabromomethazine, a New Long-Acting Sulfonamide, in Cattle. Am. J. Vet. Res., *19*, (April, 1958): 345–353.

[9] Whitlock, H. V.: Some Modifications of the McMaster Helminth Egg-Counting Technique and Apparatus. J. Council Sci. & Indust. Res., Australia, *21*, (1948): 177–180.

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Rates and Routes of Sulfonamide Excretion in the Cow
I. Milk Levels Following Single Intravenous and Oral Administrations

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THE CLINICAL APPLICATION and success of sulfonamides in veterinary therapeutics received increasing recognition in the years following their introduction, until the availability of antibiotics to the veterinary profession provided new chemotherapeutic agents that tended to replace sulfonamide therapy. However, increased evidence of bacterial resistance to antibiotics, as well as their toxicity, residual concentrations in human food products of animal sources, and their cost of administration have stimulated a renewed interest in sulfonamide therapy.

Recent information [8] indicates that a high percentage of the milk processed for human consumption contained residual antibiotics, as the result of extensive prophylactic and therapeutic intramammary medication for mastitis. This information partially indicates the need for the continued search for better professional approaches and therapy for mastitis.

Much of our present information pertaining to absorption and rates of excretion of sulfonamides for domestic animals is based on the presentation of Welsh.[9] His data emphasized the definitive variations in rates of absorption and fate of sulfonamides in different species, thus pointing to the fallacy of applying information pertaining to one species to that of another species.

Previous investigators [1,4-7] have demonstrated that sulfanilamide, sulfapyridine, sulfapyrazine, sulfadiazine, sulfamerazine, and sulfathiazole are excreted via the mammary gland in therapeutic concentrations following oral administration.

A literature review pertaining to the pharmacology, therapeutic application, and toxicity of sulfonamides in veterinary medicine has been presented by Detweiler and Scheidy.[2]

EXPERIMENTAL PROCEDURE

Sulfonamides utilized in this investigation were administered in single doses; intravenously, in 500 ml. of sterile distilled water, and orally, in the acid form, in gelatin capsules. Dosages of 0.5 and 1.0 gr. per pound of body weight were utilized for either oral or intravenous administration. Composite milk samples of all quarters of each experimental animal were collected prior to sulfonamide administration for controls. Composite milk samples of the fore milk of each quarter of each experimental animal were collected at one-, two-, four-, and eight-hour post-administration intervals. Samples collected at 12 hours and at each succeeding 12-hour interval were composites of complete milkings. All samples were refrigerated immediately following collection, and sulfonamide determinations were made within 24 hours following collection.

The sulfonamide content was determined by a modification of the Bratton Marshall method.[3] All data presented are in milligrams of free sulfonamide per 100 ml. of milk.

RESULTS

Following single intravenous administration at 1.0 gr./lb. of body weight, sulfonamides appeared in milk in the following decreasing order of concentration: sulfamethazine, sulfapyridine, sulfamerazine, sulfadiazine, and sulfathiazole (table 1). When administered at 0.5 gr./lb. of body weight, the milk concentration, in decreasing order, was as follows: sulfapyridine, sulfamethazine, sulfamerazine, sulfadiazine, and sulfathiazole (table 2).

With either of these dosages, the maximum levels usually appeared at two hours following administration, with possible therapeutic levels present within the first

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TABLE 1—Milligrams of Free Sulfonamides per 100 ml. of Milk Following Intravenous Administration of 1 gr./lb. of Body Weight

Sulfonamide	No. samples tested	Hours							
		1	2	4	8	12	24	36	48
Sulfathiazole	6								
Highest level		7.1	7.1	5.8	2.6	1.1	0.6*	0.1*	....
Lowest level		0.3	1.8	1.1	0.2	0.0	0.1	0.0	....
Average		2.7	3.5	3.1	0.1	0.3	0.2	0.02	....
Sulfapyridine	6								
Highest level		14.0	11.0	9.4	6.8	5.2	2.2	1.2	....
Lowest level		2.7	2.4	2.2	2.3	1.6	0.4	0.2	....
Average		7.5	7.6	6.7	5.1	3.6	1.5	0.7	....
Sulfamethazine	5								
Highest level		11.4	14.4	11.7†	7.4	6.2	2.8	2.8	0.9
Lowest level		6.8	7.8	6.6	4.2	2.8	0.9	0.3	0.1
Average		8.5	10.1	8.8	5.8	4.4	1.9	1.3	0.4
Sulfamerazine	5								
Highest level		9.0	10.4	11.3	4.8	3.6	0.8	0.5	....
Lowest level		1.4	2.1	3.4	1.4	0.0	0.1	0.0	....
Average		5.5	6.7	5.6	3.0	1.8	0.4	0.2	....
Sulfadiazine	3								
Highest level		5.4	6.3	5.0	3.8	0.8	0.1	0.0	....
Lowest level		1.2	1.2	1.5	1.4	0.5	0.0	0.0	....
Average		3.4	5.1	3.6	3.1	0.7	0.05	0.0	....

* Only five samples tested. † Only four samples tested.

hour following administration and remaining through the fourth hour. Sulfamethazine, sulfapyridine, and sulfamerazine presented the most persistent milk levels. Detectable concentrations of the sulfonamide compounds extended, at a maximum, over a period of 48 hours following administration.

Following a single oral administration of 1 gr./lb. of body weight, sulfonamides appeared in milk in the following decreasing order of concentration: sulfapyridine, sulfamethazine, sulfanilamide, sulfamerazine, sulfadiazine, and sulfathiazole. Sulfapyridine, sulfamethazine, sulfanilamide, and sulfamerazine presented the highest milk levels in the shortest interval following administration (4 hours) and the greatest persistence (table 3).

When administered *per os* at 0.5 gr./lb. of body weight, the sulfonamide concentration in milk was found in the following decreasing order: sulfapyridine, sulfanilamide, sulfamerazine, sulfamethazine, sulfadiazine, sulfathiazole (table 4). Sulfathiazole was usually only of detectable values and required the greatest interval following administration before being detected in the milk.

Regardless of rate of oral administration,

TABLE 2—Milligrams of Free Sulfonamides per 100 ml. of Milk Following Intravenous Administration of 0.5 gr./lb. of Body Weight

Sulfonamide	No. samples tested	Hours						
		1	2	4	8	12	24	36
Sulfathiazole	4							
Highest level		1.4	1.1	0.7	0.6	0.2	....	....
Lowest level		0.7	0.6	0.5	0.1	0.0	....	....
Average		1.0	0.9	0.6	0.4	0.1	....	....
Sulfamerazine	5							
Highest level		6.0	6.2	6.6	3.1	2.2*	0.8	0.6
Lowest level		3.4	3.8	2.9	1.8	1.2	0.4	0.1
Average		4.9	5.2	4.2	2.5	1.7	0.6	0.3
Sulfamethazine	4							
Highest level		2.9	4.4	3.9	1.9	2.0†	0.5†	0.2†
Lowest level		2.8	3.9	3.6	1.9	1.6	0.5	0.2
Average		2.9	4.2	3.8	1.9	1.8	0.5	0.2
Sulfamerazine	5							
Highest level		4.2	5.4	4.8	2.7	1.5	1.0	....
Lowest level		1.0	3.0	2.4	1.9	1.1	0.0	....
Average		2.6	4.2	3.6	2.3	1.3	0.5	....
Sulfadiazine	3							
Highest level		2.0	2.0	1.4	0.9	0.0	....	....
Lowest level		0.8	1.2	1.2	0.1	0.0	....	....
Average		1.4	1.6	1.3	0.5	0.0	....	....

* Only four samples tested. † Only three samples tested.

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TABLE 3—Milligrams of Free Sulfonamides per 100 ml. of Milk Following Oral Administration of 1 gr./lb. of Body Weight

Sulfonamide	No. samples tested	Hours							
		1	2	4	8	12	24	36	48
Sulfathiazole	5								
Highest level		0.0	0.0	0.1	0.6	0.8	0.4	0.2	....
Lowest level		0.0	0.0	0.0	0.1	0.1	0.2	0.0	....
Average		0.0	0.0	0.05	0.3	0.4	0.3	0.1	....
Sulfapyridine	5								
Highest level		0.5	1.5	4.4	5.0	6.0	5.5	3.2	1.0
Lowest level		0.1	0.2	1.4	3.2	4.3	1.7	0.8	0.4
Average		0.4	1.0	2.9	4.3	4.9	3.6	2.0	0.6
Sulfamethazine	5								
Highest level		0.4	0.9	3.0	4.6	5.3	3.8	2.5	0.8
Lowest level		0.1	0.3	2.2	3.5	3.6	1.9	1.0	0.3
Average		0.3	0.6	2.4	4.2	4.8	3.1	1.7	0.6
Sulfamerazine	6								
Highest level		0.9	1.5	4.8	7.3	7.2	4.3*	2.6	0.8
Lowest level		0.0	0.0	0.3	0.5	1.8	0.7	0.3	0.0
Average		0.3	0.5	1.3	2.4	3.0	1.7	1.0	0.2
Sulfadiazine	3								
Highest level		0.1	0.2	0.2	1.1	0.9	0.4	0.4	0.3
Lowest level		0.0	0.0	0.1	0.5	0.7	0.3	0.2	0.2
Average		0.03	0.06	0.2	0.7	0.8	0.3	0.3	0.2
Sulfanilamide	6								
Highest level		1.1	1.9	4.3	5.7	8.6	3.3	1.9	0.5
Lowest level		0.0	0.1	0.2	0.7	0.9	0.5	0.5	0.2
Average		0.2	0.7	2.1	3.5	3.9	2.2	1.2	0.4

* Only five samples tested.

milk concentration of sulfonamides appeared much later and at lower concentrations until the twelfth hour, but remained at a higher level for an additional 12 to 24 hours than when given by intravenous administration. Sulfathiazole appeared to be the least rapidly observable in the milk and in the lowest concentrations of all sulfonamides investigated.

No toxicity for any sulfonamide was observed when administered by either route or at either rate.

DISCUSSION

From the results of this investigation, it would appear that sulfapyridine, sulfamethazine, and sulfamerazine, or combinations of these sulfonamides, would be best suited for intravenous administration in bovine mastitis therapy. Probable thera-

TABLE 4—Milligrams of Free Sulfonamides per 100 ml. of Milk Following Oral Administration of 0.5 gr./lb. of Body Weight

Sulfonamide	No. samples tested	Hours							
		1	2	4	8	12	24	36	48
Sulfathiazole	3								
Highest level		0.0	0.0	0.0	0.2	0.2	0.3	0.05	0.0
Lowest level		0.0	0.0	0.0	0.05	0.2	0.05	0.0	0.0
Average		0.0	0.0	0.0	0.1	0.2	0.2	0.02	0.0
Sulfapyridine	4								
Highest level		0.05	0.7	1.7	2.7	2.6	1.6	1.0	0.2
Lowest level		0.0	0.4	1.6	2.4	1.9	1.0	0.5	0.05
Average		0.03	0.6	1.7	2.6	2.3	1.3	0.8	0.1
Sulfamethazine	4								
Highest level		0.5	0.2	0.5	0.9	0.5	0.2	0.1	....
Lowest level		0.0	0.0	0.3	0.8	0.5	0.2	0.0	....
Average		0.03	0.1	0.4	0.9	0.5	0.2	0.1	....
Sulfamerazine	5								
Highest level		0.0	0.1	1.0	1.3	1.3	0.6	0.4	0.05
Lowest level		0.0	0.05	0.6	0.8	0.8	0.4	0.1	0.05
Average		0.0	0.1	0.8	1.0	1.0	0.5	0.3	0.05
Sulfadiazine	3								
Highest level		0.3	0.1	0.3	0.6	0.6	0.6	0.2	0.0
Lowest level		0.0	0.0	0.0	0.1	0.2	0.0	0.0	0.0
Average		0.1	0.03	0.1	0.4	0.4	0.2	0.1	0.0
Sulfanilamide	6								
Highest level		0.5	0.2	0.8	2.1	2.0	1.3	0.8	0.4
Lowest level		0.05	0.2	0.6	1.7	2.0	1.1	0.6	0.2
Average		0.05	0.2	0.7	1.9	2.0	1.2	0.7	0.3

peutic concentrations of these sulfonamides were present in the milk within one hour and remained for approximately 24 hours, thus providing a rapidly available therapeutic agent, some of which would remain present in the mammary gland upon complete removal of milk at 12-hour intervals. The presence of a sulfonamide in the mammary gland while the normal routine milking process takes place would provide intramammary medication. In addition, the milking process would aid in removing the inspissated, purulent, and toxic secretion resulting from mastitis.

Data obtained following oral administration of sulfonamide compounds, at two dosage levels, indicate that those compounds best suited for intravenous administration in mastitis therapy would be of a similar value upon oral administration. However, therapeutic levels would be delayed two to four hours, compared to those following intravenous administration, but would probably remain in the milk for an additional 12 hours beyond those observed following intravenous administration. Sulfanilamide, which can only be given orally, is also found in effective concentrations in the milk.

On the basis of milk concentration as observed in this investigation, it appears that sulfathiazole would be of little value for mastitis therapy when administered either intravenously or orally.

Before individual sulfonamides and their combination can be adequately evaluated as chemotherapeutic agents for bovine mastitis following intravenous and oral administration, additional information must be obtained pertinent to spectrum of activity of sulfonamides for organisms involved in clinical mastitis, intramammary concentrations following combinations of intravenous and oral administration, and field investigations evaluating clinical results.

SUMMARY

Upon oral or intravenous administration, the sulfonamides presenting the greatest intramammary concentrations as determined by milk assay are: sulfamethazine, sulfapyridine, sulfamerazine, sulfadiazine and, only *per os*, sulfanilamide.

The concentrations of sulfathiazole found in the milk in this investigation would appear to be of little value in mastitis therapy.

Intravenous administrations provided more rapidly detectable concentrations of sulfonamides in the milk than did oral administration, while the latter provided more persistent milk levels.

References

[1] Allott, A. J.: The Treatment of Bovine Mastitis with Sulfanilamide: A Preliminary Report. J.A.V.M.A., *91*, (Nov., 1937): 588–596.

[2] Detweiler, D. K., and Scheidy, S. F.: Advances in Veterinary Science. Vol. 1. Academic Press Inc., New York, N.Y., (1953): 75–136.

[3] Fister, Harold J.: Manual of Standard Procedures for Spectrophotometric Chemistry. Standard Scientific Supply Corp., New York, N.Y., 1950.

[4] Klein, L. A., Scheidy, S. F., Kleckner, A. L., and Craige, A. H.: Studies of Sulphanilamide Action on the Cow: Preliminary Report. Univ. Pennsylvania Vet. Exten. Quart., *70*, (1938): 10–23.

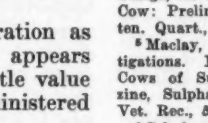
[5] Maclay, M. H., and Slavin, G.: Sulphonamide Investigations. I. The Absorption and Excretion in Dairy Cows of Sulphapyridine, Sulphamesathine, Sulphamerazine, Sulphadiazine, Sulphathiazole, and Sulphapyrazine. Vet. Rec., *59*, (1947): 313–317.

[6] Schuhardt, V. T., Carroll, T. B., Rode, L. J., and Lacy, H.: The Permeability of the Lactating Bovine Mammary Gland to Sulfonamides. Am. J. Vet. Res., *9*, (April, 1948): 144–146.

[7] Stableforth, A. W., and Hignett, S. L.: Sulphanilamide in Animals—Dosage and Tolerance. Vet. Rec., *54*, (1942): 525–532.

[8] Welch, Henry, Vester, W. R., and Burton, J. M.: Antibiotics in Fluid Market Milk, A Third National Survey. Antibiotics & Chemother., *6*, (1956): 369.

[9] Welsh, M., Schroeder, C. R., Vroman, D. F., Reddin, L., Burkhart, R., and Langer, P.: The Fate of ½ gr./lb. Body Weight of 7 Sulfonamides in 7 Animal Species. Proc. 50th Ann. Meet. U.S. Livestock San. A. (1946): 213–234.



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Udder Reactions to Sterilized Cultures and Filtrates

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QUESTIONS STILL EXIST concerning the means whereby infectious agents which invade udders produce inflammatory responses. The likelihood of allergic reactions being involved in mastitis associated with *Streptococcus agalactiae* infections was shown by Spencer and Angevine.[10] The results obtained by Pounden and Zehner[7] suggest that the low pH resulting from acid production by these organisms in the milk may be a factor.

This paper reports further attempts to explore the mechanisms involved in attacks of mastitis, associated in particular with *Str. agalactiae*, by studying clinical and histopathological reactions of experimentally produced conditions.

EXPERIMENTAL PROCEDURES

A total of 25 quarter infusions with sterilized cultures and filtrates were carried out in the preliminary series. These infusions were made into the udders of 3 aged cows and 2 younger cows. Neither of the young cows had any record of mastitis, but the 3 older cows had suffered from mastitis associated with *Str. agalactiae*.

The organisms used were *Str. agalactiae*, a hemolytic Staphylococcus, and *Cryptococcus neoformans*,[6] each obtained from a cow with mastitis, and six organisms obtained from healthy udders. These latter apparently were nonpathogenic for udders and consisted of three medium-sized rods producing yellow-colored colonies, two small diphtheroids such as are frequently observed in normal milk samples, and a nonhemolytic Micrococcus. The preparations consisted of dextrose-serum broth and raw milk cultures, bacteria-free filtrates from these, and suspensions of cells washed from agar slants with 0.4 per cent saline solution. The *C. neoformans* culture was grown in Sabourard's broth. The broth cultures, the cell suspensions, and the milk cultures were boiled to kill the organisms. Sterility checks of all the infused materials were made by culturing on blood agar.

The clinical status of the animals and their udders was closely checked during the first few hours of the postinfusion period.

In later experiments, 10 cows were used and, besides clinical observations, gross and microscopic pathology examinations were included, as the animals were slaughtered four to six hours after the udders were infused. Fourteen of the infused quarters on these cows previously had undergone attacks of mastitis produced by a variety of organisms other than *Str. agalactiae*, and an additional eight quarters had been free of mastitis or mastitis-producing organisms. These 22 quarters were infused with four different kinds of antigens prepared from *Str. agalactiae*. Two antigens were 24-hour dextrose broth cultures, one being used in the freshly prepared state and the other after boiling long enough to kill the organisms. The other two were 0.4 per cent saline solution suspensions of the organisms washed from agar slants and one of these also was boiled before using. The boiled preparations were checked for sterility as previously described.

RESULTS

When 2 to 4 ml. of dextrose-serum broth, boiled broth cultures of *Str. agalactiae*, or bacteria-free filtrates of similar cultures were infused into three previously healthy quarters there was no clinical response (table 1). The same was true of three quarters previously affected by *Str. agalactiae*, but a mild reaction occurred in an additional quarter in the same category when infused with the boiled broth culture. A

TABLE 1—Clinical Reactions Produced by Infusion of 25 Quarters with Sterilized Bacterial Cultures, Filtrates, and Cell Suspensions

Infused materials	Quantity infused (ml.)	Severity of udder reactions	
		Previously healthy	Previously mastitic
Broth, dextrose-serum	2.0	—	..
	4.0	..	—
Broth, *Str. agalactiae*	2.0	—	..
	4.0	..	+
Filtrate, *Str. agalactiae*	2.0	—	..
	3.0	..	—
	4.0	..	—
Cells, *Str. agalactiae*	4.0	..	2+
Broth, Hemolytic Staphylococcus	4.0	..	2+
Broth, Micrococcus	4.0	..	4+
Broth, medium rods	4.0	..	3+, 3+, 3+
Broth, diphtheroids	4.0	..	3+, 3+
Broth, *C. neoformans*	5.0	—,—	—,—
Milk, *Str. agalactiae*	10.0	..	3+
Milk filtrate, *Str. agalactiae*	10.0	..	3+
	12.5	..	3+
	20.0	4+	3+
	20.0	4+	..

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The technical assistance of R. K. Scherer, Animal Disease and Parasite Research Division, A.R.S., Beltsville, Md., in some of the preliminary studies is gratefully acknowledged.

moderately severe reaction, involving swelling and secretion change, occurred within three hours when an infusion was made into a different previously affected quarter using 4 ml. of a boiled washed cell suspension (nephelometer 4) of *Str. agalactiae* in 0.4 per cent saline solution.

Infusions (4 ml.) were made in seven more quarters with histories of being previously mastitic. Boiled broth cultures were used, consisting of a hemolytic Staphylococcus from a mastitic udder and the six other bacteria which had been obtained from healthy udders and which apparently were not mastitis-producing organisms. In all instances but one, a moderately severe reaction occurred within three hours and terminated the following day. The exception was a quarter which developed a severe reaction when infused with a Micrococcus preparation (table 1).

The killed *C. neoformans* culture in Sabourard's broth did not cause any visible changes when infused in 5 ml. quantities into two healthy and two previously mastitic quarters.

Moderately severe reactions developed when a 10.0 ml. quantity of sterilized *Str. agalactiae* milk culture and a 10.0, 12.5, or 20.0 ml. quantity of filtrate from milk cultures of this organism (pH 4.6) were infused into four previously affected glands. More severe reactions followed the infusion of 20.0 ml. quantities of this filtrate into two healthy quarters (table 1).

In the second series, 14 known previously affected quarters were infused, then the animals were slaughtered (table 2). Eleven of these 14 were infused with boiled, washed, 0.4 per cent saline solution suspensions (nephelometer 4), of *Str. agalactiae*, using 3.5 ml. quantities in two and 5.0 ml. in the others.

Four showed no visible change, four had mild reactions (+), and one a moderate swelling (2+), and two had severe reactions (3+ and 4+). One of the glands infused with 3.5 ml. was among those having mild reactions and the other had a moderately severe reaction. The remaining three of these 14 previously affected quarters were infused with 5.0 ml. quantities of boiled broth culture of *Str. agalactiae*, and all had severe reactions.

Fig. 1—Section from a mammary gland with previous infection, taken six hours after injection with boiled 24-hour broth culture of Streptococcus agalactiae. Notice the interstitial edema and dense leukocytic infiltration, as well as cellular and fibrinous exudate in the ducts. H & E stain; x 96.

In addition, eight healthy quarters were infused (table 3). Six of these were infused with sterilized products, two with boiled broth cultures (2 and 5 ml.), and four with boiled washed cultures (5 ml.), without visible reactions in any of them. The remaining quarters were infused with live organisms, one with a 5 ml. broth culture and the other with 5 ml. washed cell suspension (nephelometer 4), and both quarters had reacted sharply by slaughter time six hours later.

Of a total of 15 infusions made into healthy quarters in both series, four were followed by reactions and none involved

TABLE 2—Clinical Reactions from Infusion of Killed Streptococcus Agalactiae Into Lactating Udders with Previous Histories of Mastitis

No. quarters infused	Antigen used	Reactions				
		−	+	++	+++	++++
11	Washed bacteria	4	4	1	1	1
3	Broth culture	..	..	..	..	3

TABLE 3—Infusion of Streptococcus Agalactiae Into Lactating Udders with No Previous Histories of Mastitis

No. quarters infused	Antigen used	Reactions				
		−	+	++	+++	++++
2	Killed broth culture	2	..	..	..	..
4	Killed washed bacteria	4	..	..	..	..
1	Live broth culture	..	..	..	..	1
1	Live washed bacteria	..	..	..	1	..

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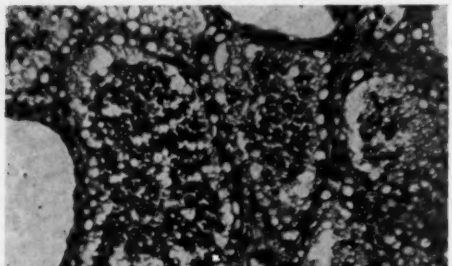

Fig. 2—Section from a mammary gland with no known previous infection, taken six hours after infection with live, 24-hour broth culture of Streptococcus agalactiae. Notice the interstitial edema and dense cellular exudate in the alveoli. H & E stain; x 144.

killed broth cultures or cell suspensions. Two were in quarters given filtered *Str. agalactiae* milk culture (pH 4.6) and two in quarters infused with live *Str. agalactiae* organisms. Reactions followed in 23 of the total of 32 infusions of sterilized bacterial cultures, filtrates, and cell suspensions into previously affected quarters.

Gross and Microscopic Pathology.—Extensive subcutaneous and interstitial edema was observed in cut sections of the infused quarters. The cut surfaces were granular and mottled grayish to dark red, but gross hemorrhage and necrosis were absent.

The histopathological changes were similar in all reacting quarters. The most noticeable and consistent changes were edema and infiltration of neutrophils into the interstitial tissue (fig. 1, 6). Lymph channels were distended with fluid which contained a few leukocytes. Neutrophils were present in heavy concentration in the interstitial tissue and they could be seen migrating through the walls of the alveoli and ducts (fig. 4). Margination of neutrophils in the blood vessels was also evident (fig. 5). In addition to neutrophils, a few lymphocytes, plasma cells, eosinophils, and monocytes were present. However, these cell types were few in number compared with the neutrophils.

Most of the alveoli and ducts were distended, although some contained milk and occasional corpora amylacea. Some alveoli were clear and open, indicating edema. Others were filled with neutrophils, fibrin, scattered plasma cells, and granular debris (fig. 1–3, 6). Some alveolar epithelial cells were vacuolated, but these cells were regularly spaced and the size of the vacuole was

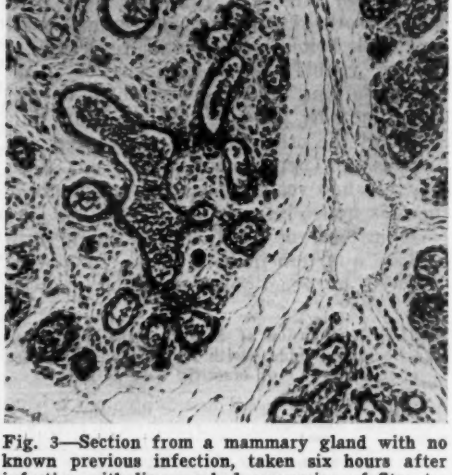

Fig. 3—Section from a mammary gland with no known previous infection, taken six hours after infection with live, washed suspension of Streptococcus agalactiae. There is heavy infiltration of leukocytes in the ducts and interstitial tissues as well as marked interstitial edema. H & E stain; x 92.

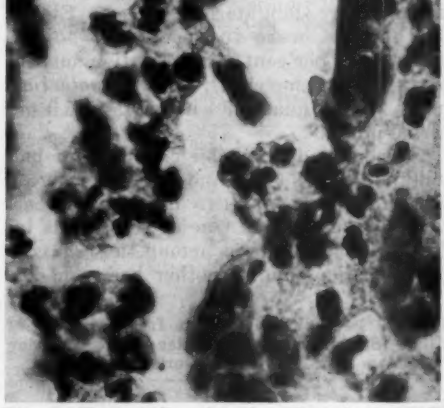

Fig. 4—Neutrophils migrating through the walls of a duct. Udder injected with boiled, 24-hour culture of Streptococcus agalactiae. H & E stain; x 972.

similar in all cells. These were considered to be functional secretory cells and not cells undergoing hydropic degeneration.

Mild degeneration was apparent in some areas which involved only the epithelial cells of the alveoli. There was no necrosis or hemorrhage.

DISCUSSION

The inflammatory reactions in these udders after infusion were clinically similar to field cases of acute mastitis. Usually, however, all indications of mastitis had disappeared after 24 hours. If the injection was repeated, a similar response occurred. The histopathology of the lesions was similar in most respects to the lesions of *Str. agalactiae* mastitis as described by others.[4,5]

The rapid reaction in previously affected udders to the presence of the killed bacterial antigens, coupled with some of the histopathological findings, indicated that hypersensitive responses occurred. The hypersensitivity to bacteria of udders that had suffered previous attacks of mastitis apparently was not limited to the organisms associated with the original attack. Udders previously affected by *Str. agalactiae* were sensitive to the infusion of several other organisms, even those not considered to be udder pathogens. Furthermore, udders previously attacked by organisms other than *Str. agalactiae* were sensitive to the presence of the latter.

The lack of response from infusion of *C. neoformans* might be considered an exception and may indicate that udder sensitivity to bacterial antigens is not broad enough to include this fungal organism. However, absence of tissue reaction in a host seems to be characteristic of infection with this organism.[1]

The response to infusions of low pH (4.6) was more intense in healthy than in previously affected udders. This may help explain the tendency for severe swelling with little or no secretions in primary attacks of *Str. agalactiae* mastitis, as compared with the effect of repeated or chronic attacks in which there frequently is a more abundant abnormal secretion but less swelling. Possibly, the presence of the streptococci is not enough to start a primary attack until their activity in milk produces a sufficiently low pH to irritate the tissues. This would then permit the injured tissues to become sensitized to bacterial components.

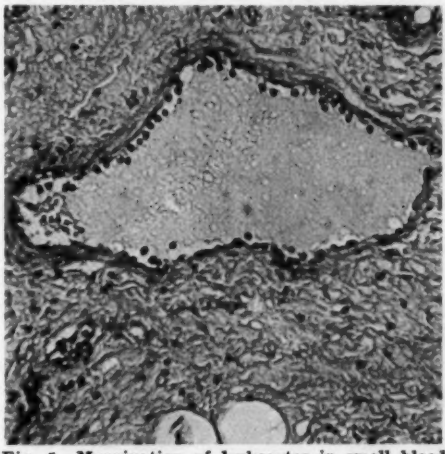

Fig. 5—Margination of leukocytes in small blood vessel in inflamed udder tissue. H & E stain; x 144.

The intense reaction within a few hours to infusion with large numbers of living *Str. agalactiae,* by the two quarters which had no previous history of mastitis seemed rapid for udders in this category. However, there probably was time for a lowering of the pH of the secretions to occur in limited areas and for this to be involved in the attack. It is also possible that these quar-

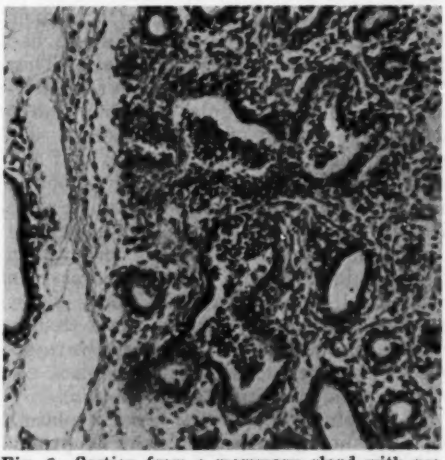

Fig. 6—Section from a mammary gland with previous infection, taken six hours after injection with a boiled, washed suspension of Streptococcus agalactiae. There is a dense infiltration of leukocytes into the interstitial spaces and ducts as well as interstitial edema. H & E stain; x 144.

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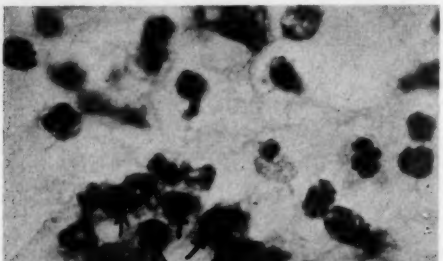

Fig. 7—Neutrophils and some plasma cells shown in inflamed udder tissue. This concentration of plasma cells (arrows) was exceptional. H & E stain; x 864.

ters had undergone unrecognized mild attacks at some previous time.

The neutrophilic response was anticipated because these cells are usually found in acute inflammations. Compared with the number of neutrophils, only a few plasma cells were seen in the interstitial spaces (fig. 7) and in the exudate. Campbell *et al.*[3] reported a heavy infiltration of plasma cells into the interstitial tissues of udders of cows that were ready to freshen, had just freshened, or when milking had been discontinued for several days. They found only scattered plasma cells in udders that were milked regularly. They stated that, "the concept of the plasmacytic origin of specific antibodies demands that any such spectacular rise in immune globulin be accompanied by a properly timed wave of plasma cell formation." They associated periods of increased antibody production in the udder with a marked increase in plasma cell population. Since only a few plasma cells were found in these tissues, either this phenomena does not always occur or else Streptococcus antigen does not stimulate much antibody production.

In veterinary pathology texts,[8,9] plasma cells are not described as being a prominent part of the cellular infiltration of inflamed mammary tissue, although some texts state that they form part of the cell population in chronic inflammation (not limited to chronic mastitis).[2,8] In the human female, a rare condition called "plasma cell mastitis" has been described but it is associated with malignancy.[2]

The rather limited plasma cell response to the presence of streptococci suggests that mechanisms other than those of an immunological nature may play a part in counteracting *Str. agalactiae* infection. A possible explanation is indicated by the noticeable reduction in the activity of this organism when it is exposed *in vitro* to abnormal milk containing serum and other products of inflammation, as compared with that in normal milk.

SUMMARY

The infusion of killed broth cultures or cell suspensions of *Str. agalactiae* into 16 quarters with previous histories of mastitis brought on attacks in 12 quarters. Similar products did not affect any of seven quarters that had no history of mastitis.

Seven quarters with previous histories of mastitis reacted when four different killed broth cultures were infused, and the same was true of four similar quarters infused with sterilized milk cultures or filtrates of *Str. agalactiae*.

Histopathologically, the reaction consisted of edema with fibrin and massive neutrophilic infiltration into the interstitial tissues, ducts, and alveoli. Only scattered plasma cells were found. These results were similar to those described for naturally occurring mastitis.

References

[1] Ainsworth, G. C.: The Pathogenicity of Fungi in Man and Animals. Mechanisms of Microbial Pathogenicity. 5th Symp. Soc. Gen. Microbiol. London, (1955): 242–262.

[2] Boyd, W.: Textbook of Pathology. 4th ed. Lea & Febiger, Philadelphia, 1946.

[3] Campbell, B., Porter, R. M., and Petersen, W. E.: Plasmacytosis of the Bovine Udder During Colostrum Secretion and Experimental Cessation of Milking. Nature, *166*, (1950): 913–914.

[4] Hemboldt, C. F., Jungherr, E. L., and Plastridge, W. N.: The Histopathology of Bovine Mastitis. Storrs [Conn.] Agric. Exper. Sta. Bull. 305, 1953.

[5] Pattison, I. H.: The Progressive Pathology of Bacterial Mastitis. Vet. Rec., *70*, (1958): 114–117.

[6] Pounden, W. D., Amberson, J. M., and Jaeger, R. F.: A Severe Mastitis Problem Associated with *Cryptococcus Neoformans* in a Large Dairy Herd. Am. J. Vet. Res., *13*, (April, 1952): 121–128.

[7] Pounden, W. D., and Zehner, C. E.: An Irritant for Udder Tissue Produced by Mastitis Streptococci in Milk. Cornell Vet., *31*, (1941): 382–388.

[8] Runnells, R. A.: Animal Pathology, 5th ed. Iowa State College Press, Ames, 1954.

[9] Smith, H. A., and Jones, T. C.: Veterinary Pathology. Lea & Febiger, Philadelphia, 1957.

[10] Spencer, G. R., and Angevine, D. M.: Pathogenesis of Bovine Mastitis. III. The Significance of Hypersensitivity in Streptococcic Infection. Am. J. Vet. Res., *11*, (July, 1950): 317–323.

Electron Microscopy of the Pulmonary Alveolar Wall

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THE STRUCTURE of the pulmonary alveolar lining has been the subject of dispute since the middle of the nineteenth century. The decisive answer to this eminently controversial issue of histology has been provided only within the last few years, by the application of the electron microscope to the study of tissues.

Although the fundamental problem of the continuity of the alveolar epithelium has been solved, a number of morphological aspects of the alveolar wall still await further elucidation. Among these are not only the fine structure of epithelial cells and boundaries between them, but also the presence of mesenchymal cellular elements in interstitial spaces of the alveolar septa. In pathological states of the lung, cells of alveolar epithelial origin appear free in the air spaces and not infrequently assume the form of vacuolated, "foamy" cells. A growing body of evidence indicates that the alveolar epithelial cells, while attached to the wall or free in the lumen, possess an appreciable degree of phagocytic power.

REVIEW OF LITERATURE

The first report on electron microscopic investigations of the lung was published in 1952 by Low.[16] The author based his findings on rat tissue fixed for four hours in a 1 per cent solution of osmium tetroxide and sectioned at the thickness of 60 to 100 mμ. The conclusions drawn by Low pointed to the existence of a continuous alveolar epithelium "with cell bodies located chiefly on the thicker portions of alveolar walls" and attenuated to form a complete lining. The thickness of the epithelial membrane was estimated at 0.1 to 0.7μ. In its very thin parts, the epithelium was thought to be "applied directly to the capillary endothelium." The "ground substance," often containing fibrillar elements and interposed between capillary endothelium and surface epithelium, was seen by Low only in some segments of the alveolar wall. The nonnucleated plaques of Kölliker[14] were not observed.

A year later, Low[17] extended his studies of the alveolar epithelium to other laboratory animals (mice, guinea pig, rabbits, and dogs) and man. He found a continuous epithelial lining in all species examined and described uninterrupted basement membranes "discretely localized along the basal surfaces of both epithelium and endothelium." Thin portions of epithelial cells were reported to contain "cytoplasmic inclusions" indistinguishable from those seen in the perinuclear moieties. Low admitted, however, that interruptions in the epithelial covering could be found at points where "free cells" were set against the wall of alveoli. Later, Low[18] suggested that in order to obtain a better preservation of cellular elements, the time of fixation with osmium tetroxide should be reduced from four hours to 60 or 30 minutes.

Early support of Low's observations came from Macklin,[20] in a paper on the pulmonary alveolar mucoid film and the pneumonocytes. Clemens[5] also confirmed the findings of Low and described a continuous membrane separating the alveolar capillaries from the air spaces. However, in 1955, Clemens[6] repudiated his earlier opinion and concluded that the alveolar epithelial cells covered only certain portions of the subjacent and continuous, structureless, basement membrane.

Swigart and Kane[40] were first to oppose the view of Low. They recognized the presence of "some material intervening between the air space and the capillary," but were not able to demonstrate the uninterrupted cellular lining. The lung tissue studied by these authors was fixed for eight hours in 1 per cent osmium tetroxide and was apparently examined under low magnifications. Essentially similar observations were made by Policard *et al.*,[27, 28] who denied the presence of a continuous alveolar epithelium, and also contended[29] that the endothelial cells of capillaries were either attenuated to a thickness of less than 10 mμ or formed a discontinuous membrane.

Two years later, however, Policard and Collet[30] recognized the continuity of the alveolar epithelium. They described it as being composed of cells possessing a small, polygonal main body and thin cytoplasmic extensions. Policard *et al.*[31] also changed their concept of the endothelium and admitted that it was continuous, although attenuated in some parts to 20 mμ. Schlipköter[33] found that the alveolar lining in the rat consisted of two layers, and reported that some of the epithelial cells contained strongly osmiophilic bodies, most of which displayed concentrically arranged internal lamellae. According to Kisch,[13] many capillaries in the alveolar walls were bare and "in direct contact with the air."

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In a preliminary report on the fine structure of pulmonary capillaries and alveoli of the mouse, Karrer [11] stated that the alveolar walls were lined by a continuous epithelial layer of an average thickness not exceeding 200 mμ. In some segments of the wall, both epithelial and endothelial cells were found to be as thin as 10 mμ. Interruptions in the epithelium, noted by Low [17] and later by Breemen and Neustein,[4] were not seen by Karrer. Septal cells were regarded by him as "bulging nucleated parts of the epithelial cells." He also described the "basement membrane" and believed that it was essentially structureless, although in some portions it apparently contained unit fibers of collagen. In a subsequent communication, Karrer [12] reaffirmed his original findings and also distinguished between two types of alveolar epithelial cells.

In extensive studies on the normal structure of lung capillaries in the frog, rat, cat, and hedgehog, Bargmann and Knoop [2] found that the alveolar epithelium was continuous and rested on a homogeneous basement membrane. Osmiophilic cytoplasmic inclusion bodies, first described by Schlipköter,[25] were regarded by Bargmann and Knoop as a product of a degenerative process, possibly of the mitochondria. Among other cellular structures observed by these workers were so-called microvilli located on the surface of some of the alveolar epithelial cells, and the endoplasmic reticulum which formed a system of tubular profiles and vacuoles. The average thickness of the cytoplasmic epithelial covering was estimated at 100 mμ.

Schulz [24] found the microvilli in the human lung not only on the main body of the alveolar cells but also on their cytoplasmic attenuations. According to this author, the epithelial cells contained, in addition to mitochondria and other cytoplasmic components perinuclear structures 2.5 μ long and 1.0 μ thick, displaying multiple, dense bands. These structures, presumably derived from mitochondria, were increased in number in lungs affected by chronic congestion as a result of mitral stenosis.

The continuity of the alveolar epithelium was further recognized in the lungs of man and the rat by Gieseking [9, 10] and in the mouse, rat, and horse by Dettmer.[5] The latter investigator, however, was not able to demonstrate a basement membrane in the alveolar wall.

Recently, Policard *et al.*[22] reaffirmed their previous views and reported that the interalveolar septa normally contain a small number of histiocytes interposed between the basement membranes of the epithelium and the capillary endothelium. Morphologically similar cells, located in the interstices of the alveolar wall and presumed to be of the connective tissue type, were observed by Schulz [26] in his study on the lung of the dormouse *(Myoxus glis)*, and by Gieseking [9, 10] in studying the lungs of man and the rat.

It is the purpose of this work to describe the ultrastructure of the pulmonary alveolar wall as observed in the rat, and to present a view on the formation of free alveolar epithelial cells.

MATERIALS AND METHODS

Ten healthy albino rats weighing approximately 100 Gm. each were used in this study.

The animals were rendered unconscious by a sharp blow on the head. As soon as was practicable, the trachea was exposed and ligated securely with a thread just below the larynx. A hypodermic needle (No. 26, ¾-in. long) was then inserted into the tracheal lumen and a sufficient amount (2 to 3 ml.) of 1 per cent buffered solution of osmium tetroxide was injected slowly into the lungs. A few minutes later, the thorax was opened and the lung removed. The parts well infused with osmium tetroxide were excised with fine scissors, cut into cubes of about 1 mm., and fixed at room temperature for 30 minutes in a 1 per cent solution of osmium tetroxide buffered with acetate veronal at a pH of 7.6.[22] The specimens were washed for 30 minutes in three changes of distilled water at 0 C. and dehydrated in ethyl alcohol of ascending grades (20, 35, 70, and 95%) at 0 C., 20 minutes in each, and in absolute ethyl alcohol, with three changes, for one hour. The tissues were then infiltrated with monomeric methacrylate (4:1 n-butyl and methyl) for two hours and embedded in prepolymerized methacrylate. After polymerization for 36 to 48 hours under ultraviolet light at 30 C., the specimens were sectioned by means of a Sjöstrand ultramicrotome equipped with a glass knife. All sections were mounted on "Athene" grids coated with a collodion film stabilized by carbon [42] and examined with an RCA, EMU 3c electron microscope.

Legends for Illustrations on Opposite Page

Fig. 1—Low power view of alveolar septum. Epithelial cell wedged between two capillaries shows cytoplasmic extensions which attenuate gradually and cover the surface of the wall. Extravasated red blood cell is present at the lower right. x 7,500.

Fig. 2—Enlarged detail of figure 1. Notice the thinness of the epithelial and endothelial extensions where they overlap the rounded edge of erythrocyte. N = nucleus, BM = basement membrane, EP = epithelial cell, EN = endothelial cell. x 30,000. Inset represents detail of figure 2. x 46,000.

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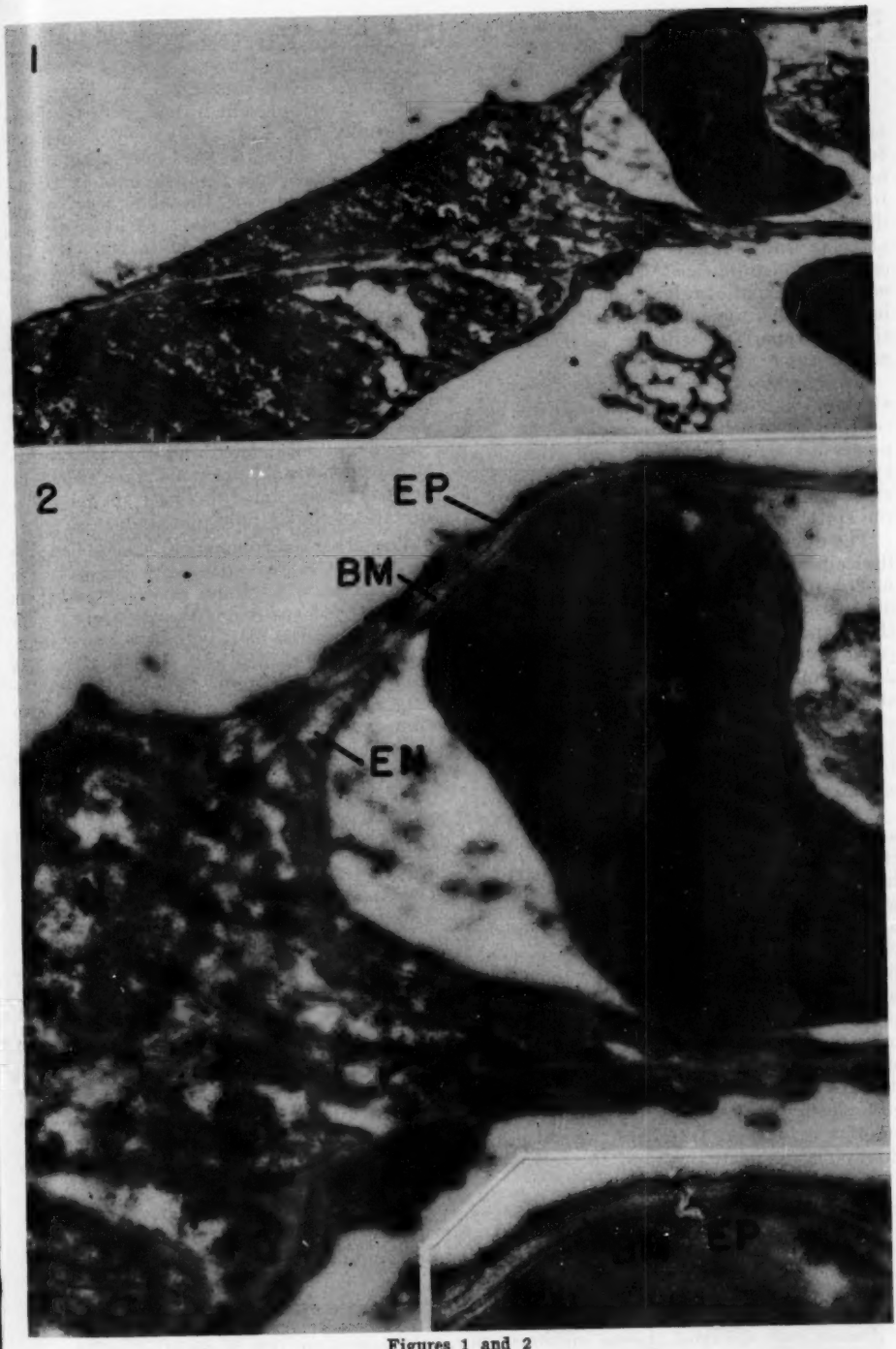

Figures 1 and 2

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OBSERVATIONS

The present study revealed that the pulmonary alveolar wall consists essentially of four elements; *i.e.*, (1) epithelium, (2) basement membrane, (3) capillary endothelium, and (4) a supporting framework of fibers. In addition to these components, the alveolar wall contains in its interstices occasional, medium-sized cells which, because of the close spatial relationship to the capillary endothelium and undetermined functional properties, will be referred to as mesenchymal cells.

Alveolar Epithelium.—The surface of the alveolar wall was found to be lined by a continuous epithelium composed of cells possessing remarkably long and attenuated cytoplasmic extensions (fig. 1–4). These extensions constituted the major part of the epithelial lining.

The nucleus-bearing moiety of the epithelial cells varied sufficiently in size and other morphological properties to allow for a distinction of two main variants. One of these, the elongated form, was usually applied to the free segment of the wall (fig. 1, 2); the other, fairly large and polygonal in shape, was located in the alveolar niche or stretched across the wall between two adjacent capillaries (fig. 4). Transitional forms between these two types were occasionally observed.

The nucleated part of the elongated variant of the cell was usually wedged between the adjoining capillaries (fig. 1, 2). In such instances, it could not be easily differentiated from the endothelial cell if examined only under a low magnification. The nucleus of this cell occupied a considerable portion of the main cytoplasmic body and was delineated by a double membrane. The perinuclear cytoplasm contained a few mitochondria displaying a characteristic structure [23,39] of internal lamellae and a double delimiting external membrane. The endoplasmic reticulum,[24] consisting of fine tubular profiles and vesicles, was also evident. The nucleated part of the cell measured 8.0 μ in length and 3.5 μ in depth (fig. 1). The cytoplasmic processes attenuated gradually (fig. 1, 2) and extended over the surface of the wall. At some points they were thin, measuring approximately 20 mμ. Generally, however, the thickness of the flattened part of the epithelium was greater, ranging from 50 to 200 mμ. The thicker segments contained a few mitochondria and a clearly identifiable network of endoplasmic reticulum. Occasionally, the surface of such segments exhibited small projections (microvilli) of varying length. The cell was enveloped by a plasma membrane, the average thickness of which was estimated as approximately 50 to 75 Å (fig. 2). Well-defined small invaginations of the membrane, previously described by Schulz,[36] were sometimes seen on the inner surface of the cytoplasmic extensions.

The other morphological variant of the epithelial cell evidently corresponds to the "septal cell" of light microscopists. Its main body measured approximately 4 μ in diameter, and in most instances was attenuated quite abruptly (fig. 3). The nucleus occupied one half to two thirds of the bulging part of the cell. In addition to numerous mitochondria and abundant endoplasmic reticulum, the perinuclear cytoplasm contained a number of fairly large (200–500 mμ long and 100–250 mμ wide) opaque bodies (osmiophilic inclusions [33]), many of which showed an irregularly laminated internal structure(fig. 3, 4, 7, 8). The surface of the cell and its adjacent, attenuated portions often displayed microvilli of an average lenth of 140 mμ.

The boundary between two adjoining epithelial cells was seen only on rare occasions. It was represented by a narrow space not exceeding 20 mμ in width and delimited by two parallel plasma membranes. The length of the junction varied greatly, depending on location. When found in thin portions of the epithelium, it usually ran a straight and short course

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Fig. 3—Epithelial cell (EP) in alveolar niche. Cytoplasmic extensions attenuate abruptly. Notice the presence of microvilli, mitochondria (M), and osmiophilic inclusions (OI), two of which show disrupted internal structure. Interstitial spaces are rich in fibrils of collagen (COL) in cross and longitudinal section and contain elastic elements (EL). A somewhat collapsed capillary with a portion of endothelial cell (EN) is also seen. x 30,000.

Figure 3

(fig. 6). In other instances, it was sinuous and of considerable length.

In 1 of the rats, occasional alveolar epithelial cells displayed pronounced and characteristic changes. The alterations occurred apparently in the osmiophilic inclusions and were characterized by an increase in number of these bodies and by disintegration of their internal structure. In advanced stages of the process, numerous sharply outlined vacuoles, containing remnants of an opaque substance, filled the major portion of the cytoplasm. The mitochondria scattered among them did not seem to be appreciably involved. The affected cells either remained attached to the wall (fig. 9), or were found evidently free in the alveolar air space (fig. 10).

Basement Membrane.—The alveolar epithelium and capillary endothelium were separated by a homogeneous membrane, the thickness of which varied from 27 mμ in its thinnest parts to approximately 200 mμ. The average thickness was estimated at 50 mμ. Areas of lighter density and ill-defined linear condensations adjoining the epithelial cells could sometimes be discerned in thicker segments of the membrane. There was, however, no evidence of particulate or fibrillar internal organization. Near the interstices of the alveolar wall which contained abundant collagenous and elastic elements (fig. 3), the basement membrane appeared to split and then followed the course of the epithelium and the endothelium.

Capillary Endothelium.—The endothelium of the alveolar capillaries was continuous and, in many structural aspects, resembled the epithelial lining. It consisted of prominent, elongated, nucleus-bearing cell bodies and their cytoplasmic extensions. The latter were usually very long and at some points attenuated to approximately 10 mμ (fig. 2). The nuclei were often indented and, generally, occupied most of the bulging part of the cell. The cytoplasm of both the cell body and the flattened extensions displayed mitochondria, a network of the endoplasmic reticulum, and a few slender surface projections (fig. 2, 3, 5). A thin (approximately 50–75 Å) plasma membrane which enveloped the endothelial cell showed, on occasion, small invaginations on the surface facing the basement membrane. At the junction of two cells, the cytoplasmic extensions were separated by a narrow cleft, well-delineated by two parallel plasma membranes (fig. 5). The junction appeared to form a direct connection between the lumen of the capillary and the basement membrane.

Framework of Fibers.—The supporting framework of the alveolar wall was represented by fibrils of collagen and elastic fibers located in the interstitial spaces. The size and distribution of these spaces varied considerably. They were most prominent at the alveolar angles (fig. 8) and in the vicinity of the alveolar sacs (fig. 11), and less common along the other segments of the wall. It seems apparent, however, that in a three-dimensional concept of the alveolus, the interstitial spaces, although frequently represented only by narrow channels, are present in many parts of the wall.

The fibrils of collagen (unit fibers of collagen [17]) were uniform in structure and had an average diameter of 15 mμ. When sectioned longitudinally, they sometimes showed a characteristic axial periodicity. The elastic fibers were represented by ir-

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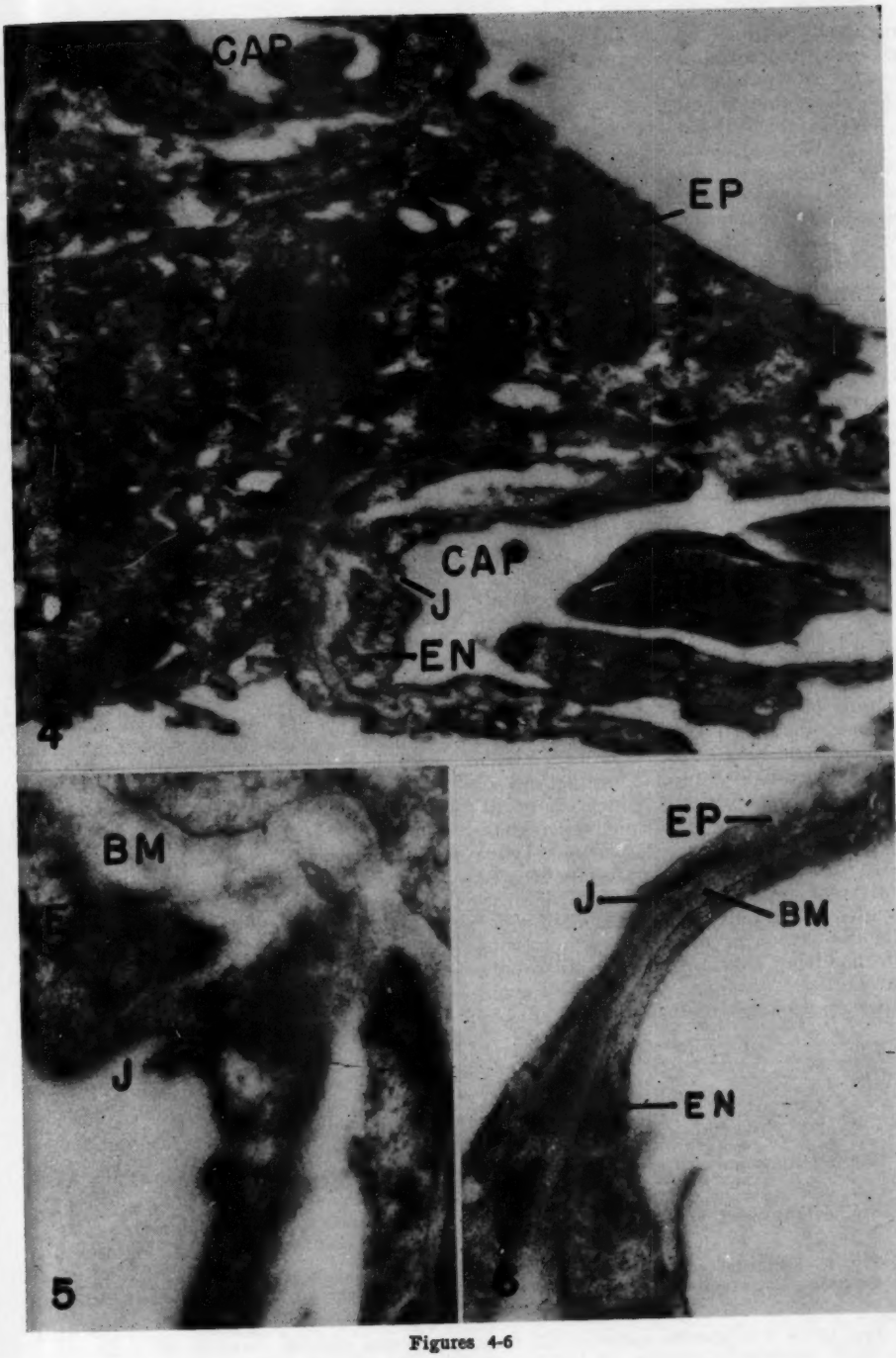
Fig. 4—Epithelial cell stretched across the wall between two capillaries. Notice abrupt cytoplasmic attenuations, mitochondria, and osmiophilic bodies, some of which are partly vacuolated. Mitochondria show small opaque granules. Boundary between endothelial cells (J). CAP = capillary, RBC = red blood cell, EP = epithelial cell, EN = endothelial cell, M = mitochondria. x 22,000.

Fig. 5—Detail of figure 4 at boundary (J). Junction between endothelial cells is represented by a narrow cleft delineated by two parallel plasma membranes. ER = tubular profile of endoplasmic reticulum, BM = basement membrane. x 65,000.

Fig. 6—Boundary (J) between epithelial cells (EP). A narrow, sharply defined cleft extends from the basement membrane (BM) to the surface. Endothelial cytoplasmic extension (EN) shows a slender projection. Linear condensation can be seen in the basement membrane. x 29,000.



Figures 4-6

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regularly outlined but well-defined, homogeneous structures of varying dimensions and of approximately the same density as the collagenous fibrils.

Mesenchymal Cells.—In addition to the collagenous and elastic elements, the interstices of the wall in proximity to alveolar sacs and possibly at alveolar intersections occasionally contained medium-sized cells with scanty cytoplasm and a relatively large nucleus. Some of these cells seemed to be embedded in a collagenous stroma (fig. 11); the others, located at alveolar angles, adjoined the epithelial and endothelial cells (fig. 12). In the latter instance, they often displayed long cytoplasmic projections wedged between the endothelium and epithelium but separated from them by the basement membrane.

The cells of this morphological type and location appeared to constitute an integral, interstitial element of the alveolar wall.

DISCUSSION

The evidence presented in this study points to the existence of a continuous epithelium in the pulmonary alveoli of the rat, and is in accordance with the findings of previous investigators. An alveolar epithelium of structurally similar character has been described in the lungs of other mammals and in the frog.[2, 8–11, 17, 30, 34, 36]

The pulmonary alveolar epithelium of the rat consists of cells which show appreciable variations in morphological characteristics. Two principal variants were distinguished: the polygonal cells located in alveolar angles and the elongated forms disposed usually along the wall. The former cells displayed, in addition to numerous mitochondria and prominent microvilli, characteristic opaque, round, or ovoid bodies (osmiophilic inclusions) scattered in the cytoplasm. Such bodies were not seen in the elongated type of alveolar cell. Structural differences between these two variants were confined only to the perinuclear part of the cells; the cytoplasmic extensions possessed identical morphological organization. While the significance of these differences is not clear, it would seem reasonable to infer from the occurrence of transitional forms of epithelial cells that the variations in structure may reflect the influence of location rather than the diversity of function. This view, although entirely conjectural, seems to be supported by the common observation that under conditions of cellular stimulation; *e.g.*, by viral and some other agents, apparently all variants of epithelial cells hypertrophy and proliferate essentially to the same degree, often giving rise to the formation of a distinct and uniform epithelial membrane. This process of "epithelialization" is strikingly illustrated in pulmonary adenomatosis of sheep (jagziekte)[7] and in a number of viral[3, 15, 25, 26, 41] and rickettsial[1] pneumonias. The phenomenon of alveolar "epithelialization," as observed with the light microscope, will be the subject of a separate communication.

Morphological variants of the cells lining the pulmonary alveoli have been previously mentioned by Karrer[11] and recognized by Policard *et al.*[32] The latter authors described the alveolar epithelium as being composed essentially of small elements of the lymphocytic type with long cytoplasmic extensions, and of larger cells which do not attenuate to take part in the formation of the lining but give rise, under pathological conditions, to vacuolated intra-alveolar cells. The presence of such large cells without cytoplasmic extensions was not revealed by this study.

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Fig. 7—Epithelial cell showing mitochondria (M), and partly disrupted osmiophilic inclusions. x 32,000.

Fig. 8—Disrupted osmiophilic inclusions (OI) and apparently normal mitochondria (M) in alveolar epithelial cell. x 20,000.

Fig. 9—Epithelial cell contains numerous vacuoles showing the remnants of osmiophilic material. Extravasated erythrocyte is seen at top. N = nucleus, M = mitochondria, EN = endothelial cell. x 7,500.

Fig. 10—A free alveolar epithelial cell showing numerous vacuoles, many of which contain osmiophilic material. A few mitochondria (M) can also be seen. x 10,500.

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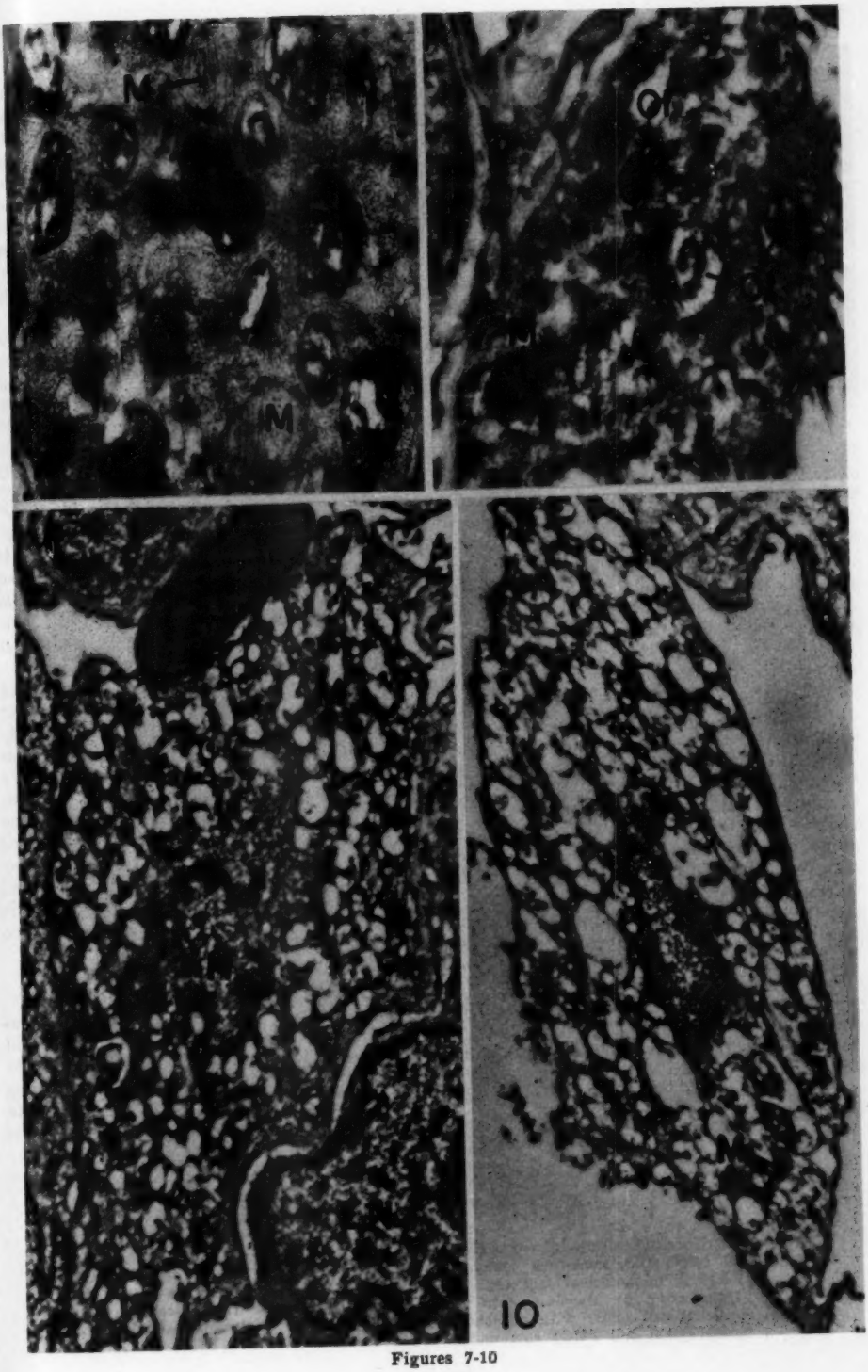

Figures 7-10

The mode of formation and functional significance of the osmiophilic inclusions are not clearly understood. Bargmann and Knoop [2] thought that they represent a degenerate form of mitochondria. A view maintaining that these two structures differ sufficiently in morphological characteristics to be regarded as separate entities was recently put forward by Schulz.[38] No transitional forms between mitochondria and osmiophilic inclusions were observed by the present authors in the normal lungs of the rat. In this connection, it is of interest that the degenerative processes in mitochondria are characterized by swelling and disintegration of cristae,[21] or by vacuolization of the matrix and condensation of internal lamellae with the subsequent formation of opaque, transverse bands.[37] The latter changes were found to be quite marked under conditions of hypoxia and increased oxygen tension.[35,36]

The disruption of osmiophilic bodies was pronounced in occasional alveolar epithelial cells in one of the lungs studied. The involved cells showed a great number of vacuoles in which the remnants of peripherally distributed osmiophilic material could often be observed. Mitochondria of apparently normal internal structure were seen scattered among the vacuolated areas. The significance of such vacuoles is not known. Macklin [20] suggested, in a paper on the mucoid film covering the pulmonary alveoli, that the so-called "granular pneumonocytes" serve as "the originators of the secretion which composes the film." This worker also distinguished other forms of the alveolar epithelial cells: phagocytic and membranous. All three types were regarded by him as "specializations of the original entodermal epithelium of the lung."

Low and Sampaio [19] were inclined to regard the alveolar epithelial cells as entodermal in origin, but denied that they possess phagocytic powers. Phagocytosis of particles of gold, silver, mercury, and iron by alveolar epithelial cells has recently been described and supported by convincing pictorial evidence by Gieseking.[10] The author demonstrated that the ingested particles could be found principally in the vacuoles, and to some extent in the mitochondria. Larger particles, such as carbon and crystals of hemosiderin, were shown by him to remain free in the cytoplasmic matrix. The transformation of alveolar epithelial cells into macrophages has also been reported by Schulz.[38]

Of considerable interest is the presence of cellular elements in the interstitial spaces of the alveolar wall. In the normal lung, these cells are quite sparse and, on occasion, seem to be closely associated with collagenous fibrils (fig. 11). Their origin and functional properties are not clearly understood. Apparently identical cells have been described recently by Policard *et al.*[32] as histiocytes, and by Schulz [36] and Gieseking [9,10] as connective tissue elements. It is felt, however, that more evidence based on experimental procedures and on serial sections is needed to determine the true identity, especially of the cells located at intersections of the alveolar walls (fig. 12).

In a random section, a cell belonging either to the endothelium of an adjacent capillary or to the epithelium of an alveolus can appear isolated inside an alveolar septum and completely surrounded by a basement membrane. Accordingly, the criteria used in this work for identification of such cells are only tentative and based on the presence of long cytoplasmic processes wedged between the epithelium and the endothelium. Further studies, now in progress, should provide more information on the character of these cells.

SUMMARY

Lung tissue from 10 albino rats was fixed for 30 minutes in a 1 per cent buffered solution of osmium tetroxide, dehydrated in ethyl alcohol of ascending grades, and embedded in methacrylate. After polymerization under ultraviolet

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Fig. 11—A free segment of the alveolar septum. Notice large tissue space containing a single mesenchymal cell (MC), numerous collagenous fibrils (COL), and opaque, elastic elements (EL). A mononuclear leukocyte (L) is present in the capillary. x 9,500.

Fig. 12—Intersection of alveolar septa showing endothelial cells (EN), capillary lumen (CAP), epithelial cell (EP) with mitochondria and osmiophilic inclusions, and presumably a mesenchymal cell (MC) surrounded by basement membrane (BM). x 9,500.

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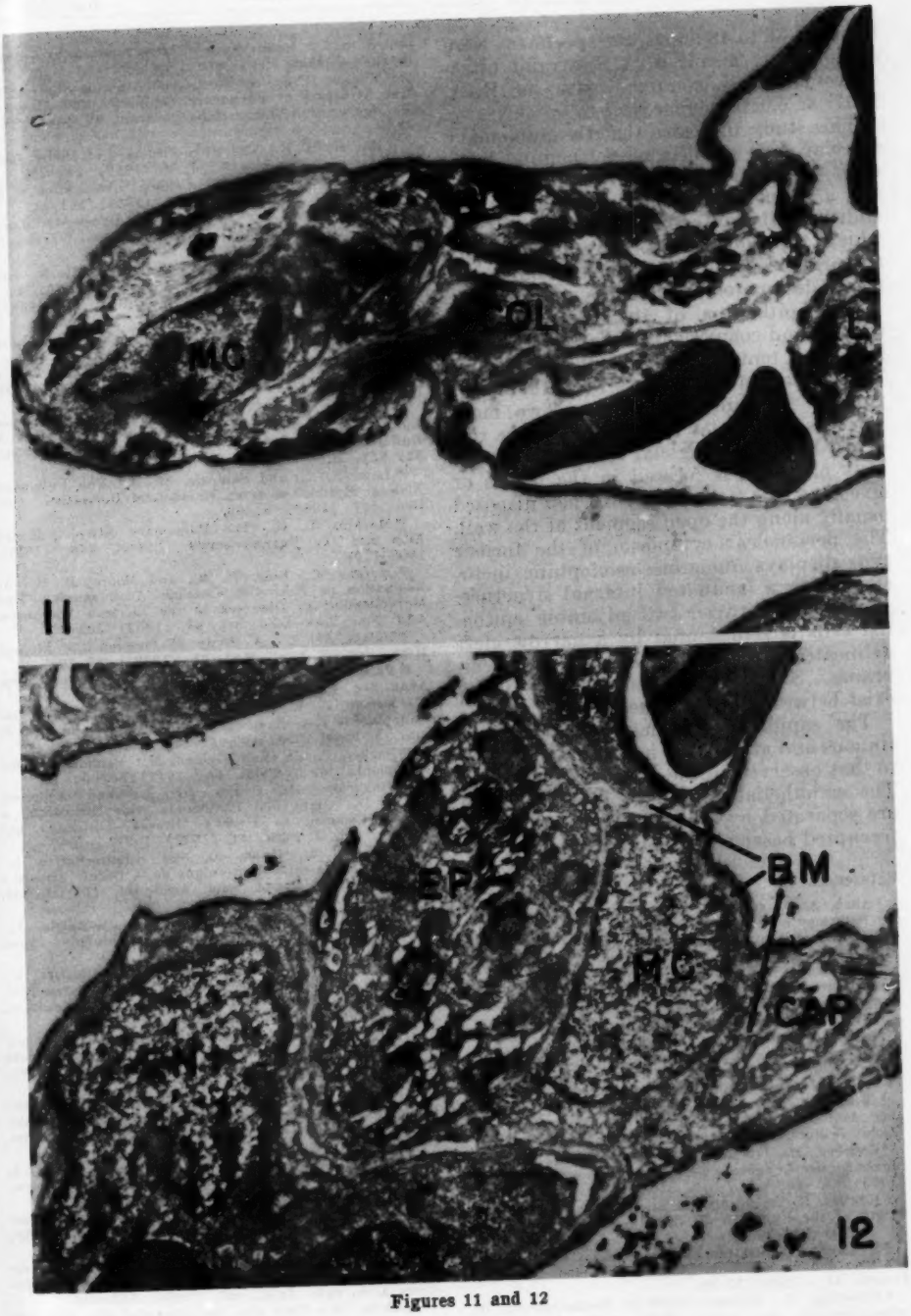

Figures 11 and 12

light for 36 to 48 hours, the specimens were sectioned by means of a Sjöstrand ultramicrotome and examined with an RCA, EMU 3c electron microscope.

This study indicates that the pulmonary alveolar wall consists of epithelium, basement membrane, capillary endothelium, and a supporting framework of fibers. In addition, the interstices of the wall, in which the collagenous and elastic fibers are present, contain occasional cellular elements designated as mesenchymal cells.

The epithelium of the alveoli is continuous and composed of cells which possess long and tenuous cytoplasmic extensions. These attenuated processes form the major part of the epithelial lining. Two main variants of alveolar epithelial cells are recognized: (1) polygonal cells, the main body of which is located in the niches of alveoli, and (2) elongated forms, disposed usually along the open segment of the wall. The perinuclear cytoplasm of the former type displays numerous osmiophilic inclusions with a laminated internal structure. A junction between two adjoining epithelial cells is represented by a narrow cleft delineated by two parallel plasma membranes. Structurally identical boundaries exist between the endothelial cells.

The capillary endothelium is also continuous and attenuated in a manner similar to that observed in the alveolar epithelium. The endothelial and the epithelial linings are separated by a homogeneous and uninterrupted basement membrane.

References

[1] Allen, A. C., and Spitz, S.: A Comparative Study of the Pathology of Scrub Typhus (Tsutsugamushi Disease) and Other Rickettsial Diseases. Am. J. Path., *21*, (1945): 603–681.

[2] Bargmann, W., and Knoop, A.: Vergleichende elektronenmikroskopische Untersuchungen der Lungenkapillaren. Ztschr. Zellforsch., *44*, (1956): 263–281.

[3] Bienfet, V.: Les Pneumonies Interstitielles à Cellules Géantes. Ann. méd. vét., *97*, (1953): 51–62.

[4] Breemen, V. L., and Neustein, H. B.: Electron Microscopic Studies of Lung Tissue from Guinea Pigs and Premature Infants. Anat. Rec., *124*, (1956): 472–473.

[5] Clemens, H. J.: Elektronenoptische Untersuchungen über den Bau der Alveolenwand in der Rattenlunge. Ztschr. Zellforsch., *40*, (1954): 1–7.

[6] Clemens, H. J.: Elektronenmikroskopische Beobachtungen an der Lungenalveole. Gegenbaurs morph. Jb., *94*, (1955): 471–492.

[7] Cowdry, E. V.: Studies on Etiology of Jaagsiekte. II. Origin of the Epithelial Proliferations. J. Exptl. Med., *42*, (1925): 334–345.

[8] Dettmer, N.: Elektronenmikroskopische Untersuchungen über den Wandbau der Lungenalveole. Beitr. Silikose-Forsch., *45*, (1956): 17–42.

[9] Gieseking, R.: Elektronenoptische Beobachtungen im Alveolarbereich der Lunge. Beitr. path. Anat., *116*, (1956): 117–119.

[10] Gieseking, R.: Aufnahme und Ablagerung von Fremdstoffen in der Lunge nach elektronenoptischen Untersuchungen. Ergebn. Path., *38*, (1958): 92–126.

[11] Karrer, H. E.: An Electron Microscopic Study of the Fine Structure of Pulmonary Capillaries and Alveoli of the Mouse. Bull. Johns Hopkins Hosp., *98*, (1956): 65–83.

[12] Karrer, H. E.: The Ultra-Structure of Mouse Lung. J. Biophys. & Biochem. Cytol., *2*, (1956): 241–252.

[13] Kisch, B.: Electron Microscopic Investigation of the Lungs. Exptl. Med. & Surg., *13*, (1955): 101–117.

[14] Kölliker, A.: Zur Kenntnis des Baues der Lunge des Menschen. Verhandl. phys.-med. Ges. Würzb., *16*, (1881): 1–24.

[15] Lauder, I. N., Martin, W. B., Gordon, E. B., Lawson, D. D., Campbell, R. S. F., and Watrach, A. M.: A Survey of Canine Distemper. Vet. Rec., *66*, (1954): 623–631.

[16] Low, F. N.: Electron Microscopy of the Rat Lung. Anat. Rec., *113*, (1952): 437–450.

[17] Low, F. N.: The Pulmonary Alveolar Epithelium of Laboratory Mammals and Man. Anat. Rec., *117*, (1953): 241–264.

[18] Low, F. N.: The Electron Microscopy of Sectioned Lung Tissue after Varied Duration of Fixation in Buffered Osmium Tetroxide. Anat. Rec., *120*, (1954): 827–851.

[19] Low, F. N., and Sampaio, M. M.: The Pulmonary Alveolar Epithelium as an Entodermal Derivative. Anat. Rec., *127*, (1957): 51–63.

[20] Macklin, C. C.: The Pulmonary Alveolar Mucoid Film and the Pneumonocytes. Lancet, *266*, (1954): 1099–1104.

[21] Morgan, C., Rose, H. M., and Moore, D. H.: An Evaluation of Host Cell Changes Accompanying Viral Multiplication as Observed in the Electron Microscope. Ann. New York Acad. Sci., *68*, (1957): 302–323.

[22] Palade, G. E.: A Study of Fixation for Electron Microscopy. J. Exptl. Med., *95*, (1952): 285–298.

[23] Palade, G. E.: The Fine Structure of Mitochondria. Anat. Rec., *114*, (1952): 427–451.

[24] Palade, G. E., and Porter, K. E.: Studies on the Endoplasmic Reticulum. I. Its Identification in Cells *in Situ*. J. Exptl. Med., *100*, (1954): 641–656.

[25] Parker, F., Jr., and Finland, M.: Primary Atypical Pneumonia. Arch. Path., *44*, (1947): 581–608.

[26] Pinkerton, H., Smiley, W. L., and Anderson, W. A. D.: Giant Cell Pneumonia with Inclusions. A Lesion Common to Hecht's Disease, Distemper and Measles. Am. J. Path., *21*, (1945): 1–23.

[27] Policard, A., Collet, A., and Giltaire-Ralyte, L.: Étude au Microscope Électronique de la Cellule Alvéolaire de Mammifère. Compt. rend. Acad. sci. (Paris), *239*, (1954): 573–575.

[28] Policard, A., Collet, A., and Giltaire-Ralyte, L.: L'Alvéole Pulmonaire au Microscope Électronique. Presse méd., *62*, (1954): 1775–1777.

[29] Policard, A., Collet, A., and Giltaire-Ralyte, L.: Étude au Microscope Électronique des Capillaires Pulmonaires chez les Mammifères. Compt. rend. Acad. sci. (Paris), *239*, (1954): 687–689.

[30] Policard, A., and Collet, A.: Apports de la Microscopie Électronique à la Connaissance Histo-Physiologique de la Paroi Alvéolaire. J. Physiol., Paris, *48*, (1956): 687–690.

[31] Policard, A., Collet, A., and Pregermain, S.: Sur Quelques Dispositions Inframicroscopiques de l'Endothélium des Capillaires Pulmonaires chez les Mammifères. Compt. rend. Acad. sci. (Paris), *242*, (1956): 8–11.

[32] Policard, A., Collet, A., and Pregermain, S.: Étude au Microscope Électronique des Capillaires Pulmonaires. Acta. Anat., *30*, (1957): 624–638.

[33] Schlipköter, H. W.: Elektronenoptische Untersuchungen ultradünner Lungenschnitte. Deutsch. med. Wchnschr., *79*, (1954): 1658–1659.

[34] Schulz, H.: Elektronenoptische Untersuchungen der normalen Lunge und der Lunge bei Mitralstenose. Virchows Arch. path. Anat., *328*, (1956): 582–604.

[35] Schulz, H.: Über den Gestaltwandel der Mitochondrien im Alveolarepithel unter CO_2- und O_2-Atmung. Naturwissenschaften, *43*, (1956): 205–206.

[36] Schulz, H.: Elektronenmikroskopische Untersuchun-

gen der Lunge des Siebenschläfers nach Hibernation. Ztschr. Zellforsch., *46*, (1957): 583–597.

[37] Schulz, H.: Die Pathologie der Mitochondrien im Alveolarepithel der Lunge. Beitr. path. Anat., *119*, (1958): 45–70.

[38] Schulz, H.: Die submikroskopische Pathologie der Cytosomen in den Alveolarmakrophagen der Lunge. Beitr. path. Anat., *119*, (1958): 71–91.

[39] Sjöstrand, F. S., and Rhodin, J.: The Ultrastructure of the Proximal Convoluted Tubules of the Mouse Kidney as Revealed by High Resolution Electron Microscopy. Expti. Cell Res., *4*, (1953): 426–456.

[40] Swigart, R. H., and Kane, D. J.: Electron Microscopic Observations of Pulmonary Alveoli. Anat. Rec., *118*, (1954): 57–71.

[41] Watrach, A. M.: Ph.D. thesis, University of Glasgow, Scotland, 1957.

[42] Watson, M. L.: The Use of Carbon Films to Support Tissue Sections for Electron Microscopy. J. Biophys. & Biochem. Cytol., *1*, (1955): 183–184.

Activity in Milk of a Mastitis-Producing Staphylococcus, a Coliform Organism, and *Streptococcus Agalactiae*

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PREVIOUS WORK has shown that milk varies in its ability to resist the action of *Streptococcus agalactiae*.[3-7] The procedure of exposing milk samples to the action of *Str. agalactiae* and measuring their relative resistance has not been considered a test for resistance of the cows to mastitis. The observations, however, have had value in drawing attention to factors which may be influencing manifestations of this disease complex. They helped materially, for instance, in developing the study which demonstrated a greater tendency for attacks to occur in cows fed freshly cut legume-grass forage, as opposed to feeding it as silage.[8]

This report concerns a procedure used to explore variation in milk samples to the action of two other mastitis-producing bacteria, staphylococci and coliform organisms. The relative ability of samples to "poise" or maintain an oxidation-reduction (O/R) equilibrium against the reducing action of these organisms formed the basis of the method. During preliminary studies leading to the use of this procedure, the O/R potentials of numerous milk samples were determined. There appeared to be some influence on these during the change of cows to pasture and, consequently, data on this aspect also are presented.

MATERIALS AND METHODS

Quarter milk samples were collected weekly from Holstein-Friesian and Jersey cows. All animals were selected on the basis of freedom from mastitis and having recently freshened. In order to maintain a continuity of the experiments from year to year, it was necessary to substitute animals as time passed. Two groups of cows were used. Group 1 contained an average of 12 lactating cows. Cows in this group were fed the usual winter ration of relatively good quality legume-grass hay, grain, and either forage crop or corn silage. Pasture was provided in season, along with moderate quantities of grain. Group 2 contained an average of only 5 lactating cows. These animals were given dry feed alone consisting of medium to poor quality mixed hay and grain.

The milk samples were collected aseptically in the morning within two hours after the animals were milked. Separate portions of each sample were used for inoculation with each of the three cultures—a Staphylococcus, a coliform organism, and *Str. agalactiae*. These cultures had been isolated from udders during attacks of mastitis. The Staphylococcus was a strain that produced relatively wide zones of *alpha* and *beta* hemolysis and was coagulase positive. The coliform organism was *Escherichia coli*, based on its biochemical reaction. The *Str. agalactiae* was the same strain used in previous work.[7]

In setting up the Staphylococcus and coliform tests, 0.25 ml. of a sterile 1:1,000 aqueous solution of methylene blue chloride and 10.0 ml. of milk were used. The dye was put in sterile test tubes and followed by the addition of the milk. The procedure for *Str. agalactiae* used brom cresol purple and was conducted as previously described.[7] To inoculate the tubes with the staphylococci and coliform organisms, a loop (3 mm. inside diameter of 24-gauge wire) of 24-hour dextrose broth culture was used. The tubes were then placed in the incubator and hourly readings were made for eight hours to check the rapidity with which the dye was reduced, as shown by the loss of the blue color. Changes in this respect were recorded by using ratings. These were determined arbitrarily by assigning figures of 1+ to 4+. A tube which had as much as a definite white streak down one side was given a 1+ rating. When the solution had turned approximately half white, it was given a 2+ rating; when three fourths became white, 3+; and where all the blue had disappeared, a 4+ rating. In practice, the initial change of 1+ or more was found to be the most suitable for making comparisons and, thus, is the one used in this report. Tests which failed to undergo any loss in color within the eight-hour observation period were assigned a figure of ten hours for calculation purposes.

In determining O/R potentials of milk samples, a pH meter* and a gold electrode, thimble style,†

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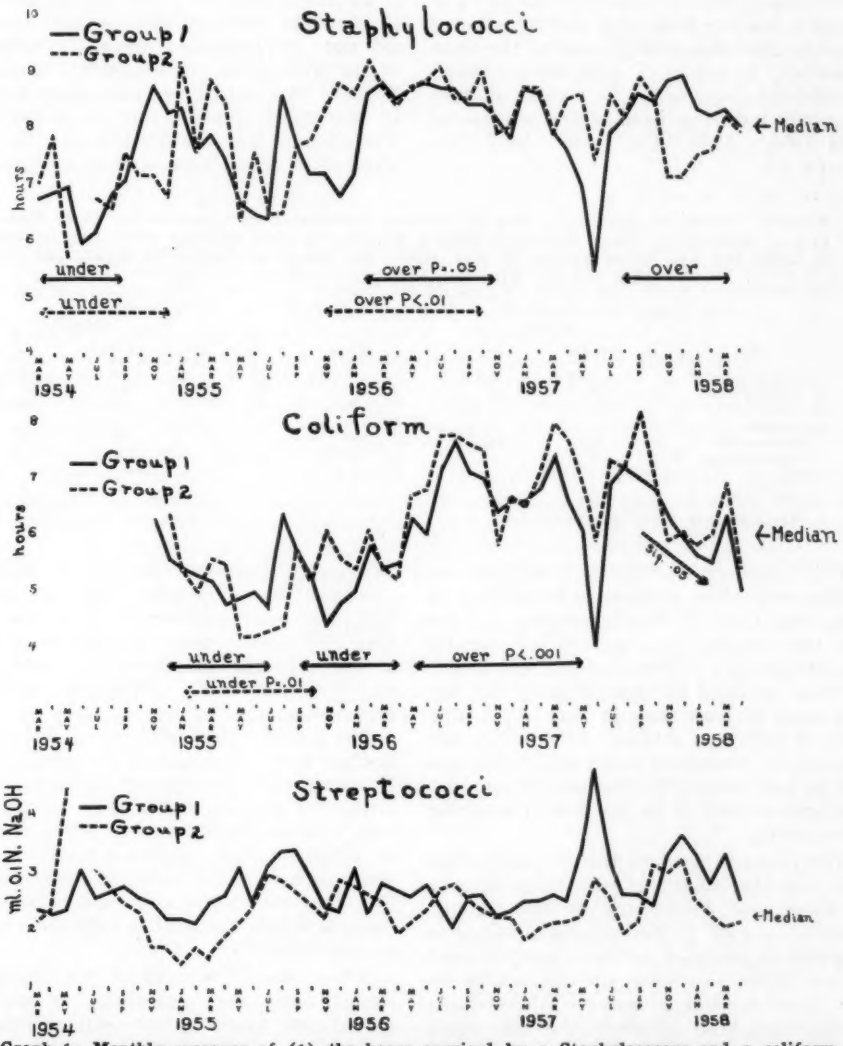
* Beckman pH meter, model H, series 1088 and † Beckman gold electrode, thimble style, model 1275, manufactured by Beckman Instruments Inc., South Pasadena, Calif.

were used. The procedure followed was that outlined in the bulletin supplied by the manufacturer. The reading point used was that existing five minutes after putting the electrode in the milk. This interval was chosen because drifting generally had reached a practical minimum by then.

RESULTS

The trend of the number of hours required for Staphylococcus to produce the initial change in color is shown in graphic form by monthly averages for the two groups of cows (graph 1). The periods



Graph 1—Monthly averages of (1) the hours required by a Staphylococcus and a coliform organism to reduce the oxidation-reduction potential of milk samples as shown by loss of color of methylene blue, and (2) the milliliters of NaOH required to neutralize acid produced in similar samples by Streptococcus agalactiae. The notations concern the median and significant runs of monthly average serial readings above and below it. The cows in group 1 were fed hay, grain, and silage or pasture in season and those in group 2 were fed hay and grain alone.

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when reduction occurred most rapidly in samples from cows in group 1 that were fed hay, grain, silage, or pasture in season were June and July of 1954, May, June, and July of 1955, and June of 1957. In December, 1955, and in the months before and after it, a shorter reduction time was also encountered, but it was not so pronounced. The averages of the cows in group 2, fed dry feed only, closely followed those in the other group most of the time. However, a tendency existed for their monthly averages to be the reverse of those in group 1 during part of the winters of 1954–1955, 1955–1956, and 1957–1958 (graph 1).

was relatively suitable for, or lacked resistance to, activity by the three organisms. Opposed to this, the results in August, 1955, showed that while the milk samples were fairly resistant to the action of Staphylococcus and the coliform organism, they were relatively lacking in resistance to Streptococcus.

To detect whether or not the fluctuations in the data were random, several statistical tests were applied using the numbers of the runs' consecutive monthly averages. Tests of this nature are commonly applied in statistical quality control procedures. The medians were established and the total number of runs counted that contained a

TABLE 1—Runs of Consecutive Monthly Average Determinations Obtained for Three Measures of Activity of Three Mastitis-Producing Bacteria in Milk Samples from Two Groups of Cows, One Fed Mixed Rations of Hay, Grain, and Silage or Pasture in Season and the Other Fed Dry Feed Only, Composed of Hay and Grain

	Staph. aureus		*Esch. coli*		*Str. agalactiae*	
Item	Mixed	Dry	Mixed	Dry	Mixed	Dry
Median	8.05	8.05	6.05	6.05	2.6	2.3
No. observations	50	48	42	40	40	46
No. runs	11*	15*	10*	14*	15*	13*
Longest run						
Over median	10†	12*	13*	6	5	5
Under median	7	9	8	10*	6	8
Longest run up or down	5	4	6†	4	3	5

* Significant at 0.01; † significant at 0.05.

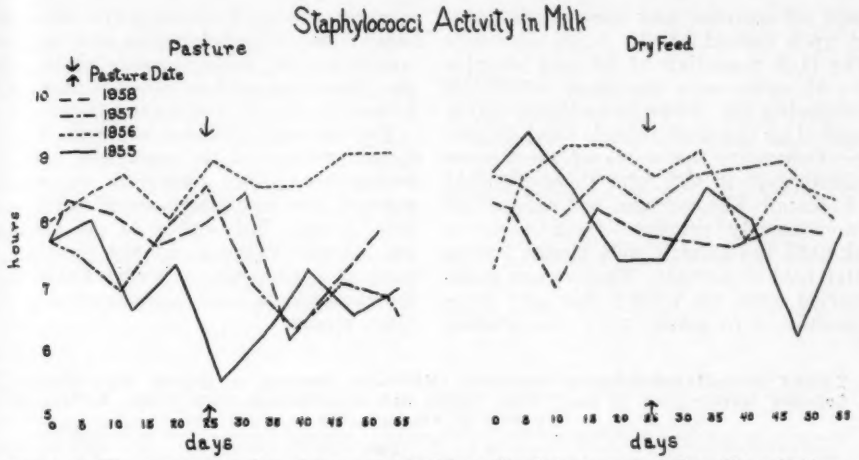
The tendency was for the coliform organism reduction periods to be shorter in time than those of Staphylococcus and for the test results to often follow similar trends (graph 1). For example, the length of time required for reduction of the dye was short in both tests in May, June, and July of 1955 and June of 1957. However, there were numerous times when this was not so, and greater fluctuations in monthly averages seemed to be the rule during the later years.

The measure used in the *Str. agalactiae* test was the ability of the milk samples to resist acid production by the streptococci. A review of the results obtained in this test on parts of the same samples used in the other two tests showed tendencies for these to follow trends in resistance similar to those obtained for the other organisms (graph 1). For example, this was true for the averages for June, 1957, when acid production by *Str. agalactiae* was high and reduction times short in the other two tests, all indicating the milk

minimum of seven consecutive monthly averages. In every case, with one exception, the total number of runs was less than one would expect by chance (1 time in 100). In the exception, the probability was less than 0.05.[9] This test indicated that nonrandom influences were at work.

The longest runs above and below the median were counted and are given (table 1) along with appropriate significance notations.[2] Following the quality control procedure of accepting runs of seven or more as indicative of a significant trend, runs of seven or more have been indicated (graph 1). They may serve as an aid in selecting times in which nonrandom influences might be operating.

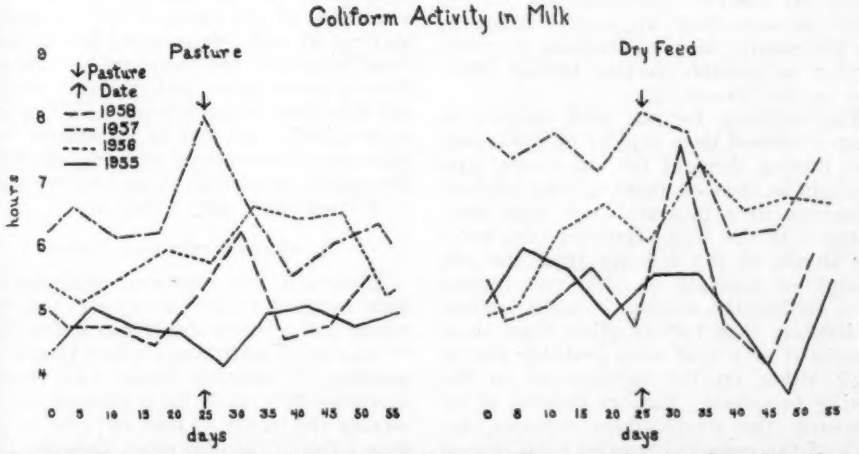
When weekly results of the Staphylococcus tests were examined, it was observed that losses by the milk samples in resistance to the activity of these organisms sometimes occurred on turning cows on pasture in similar manner to that observed for *Str. agalactiae*.[6] A comparison of four years of weekly averages showed



Graph 2—The average number of hours required by a Staphylococcus to reduce the oxidation-reduction potential of milk samples as shown by a loss of color of methylene blue before and after a group of cows was changed from rations of hay, grain, and silage to pasture and another group that was fed hay and grain throughout the experiment.

that in one year (1955) a marked drop in reduction time occurred within two days after the animals went on pasture (graph 2). In two other years (1957 and 1958), a drop in the reduction time was observed in the tests two weeks after the animals were turned to pasture. The resistance level increased somewhat after the initial drop. In 1956, no lowering occurred. Samples collected during the same period from cows that did not go on pasture showed less change (graph 2), although in 1955 rather erratic results were obtained in the weekly results just prior to the pasture date.

Tendencies for changes in resistance in the weekly coliform organism tests that could be associated with the change to pasture were not discernible (graph 3). The erratic nature of the test results for both



Graph 3—The average number of hours required by a coliform organism to reduce the oxidation-reduction potential of milk samples as shown by a loss of color of methylene blue before and after a group of cows was changed from rations of hay, grain, and silage to pasture and another group that was fed hay and grain throughout the experiment.

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groups of samples may have partly covered up a limited effect.

The O/R potentials of 48 milk samples from 12 cows were measured weekly in 1954 during the weeks immediately prior to and after the time animals went to pasture. Generally, the range of the figures obtained were in line with those reported by Jackson.[1] The averages and ranges had been relatively constant from week to week until the animals were changed from winter feed to pasture. The average measurements made the week following change to pasture were lower (table 2). During the three weeks following, the average measurements tended to return to the same level as during the prepasture period.

TABLE 2—Oxidation-Reduction Potentials (Millivolts) Obtained on Quarter Milk Samples Collected Weekly from 12 Dairy Cows Before and After Change from Winter Rations to Pasture on April 16, 1954

Item	Date							
	4/8	4/15	4/23	4/29	5/6	5/13	5/27	6/2
Average	238.49	235.87	227.00	234.76	231.11	233.99	229.99	229.90
Range	224.55 to 245.3	224.30 to 250.55	213.80 to 241.55	222.65 to 243.05	225.05 to 234.8	223.3 to 241.8	219.05 to 247.3	218.3 to 241.8

Discussion

These tests can not be considered as measures of resistance to mastitis any more than can the procedure used with *Str. agalactiae*. However, they provide opportunity to observe environmental factors which, because they apparently influence the test results, may be desirable to study further as possible factors having influence on the disease.

The tendency for the milk samples to become reduced more rapidly by the organisms during three of four summers, particularly in cows on pasture, may indicate influences were present which were associated with the feed. However, the varying trends of the samples from the two groups of animals on different rations were sufficiently similar in some periods to indicate that factors other than those associated with feed were probably also at work either on the animals or on the testing procedure. Factors capable of influencing the relationships between the milk of the cows fed regular herd rations and the bacteria used in these three tests apparently reached an unusual intensity in the early summer of 1957. The lowest monthly average times required for reduction of the Staphylococcus and coliform organism tests were encountered in June, the time when acid production was at its highest in the Streptococcus tests.

The manner in which the milk reacted to the presence of the organisms from one period to another appeared to resemble some of the manifestations of mastitis in dairy herds. This refers to the tendency for marked increases of the problem to occur in some herds during limited periods, for example, as those mentioned in a previous report.[8]

Exposure of the samples to air during collection and conduct of the challenge might be considered as getting away from actual conditions in udders and could be sufficient to influence the results of the tests. However, as the test is one of "poising," this should not have a significant effect. Furthermore, the difference between anaerobically and aerobically collected samples as far as their ability to bring about loss of the color of methylene blue may not be as much as might be concluded from reports in the literature.[1,10] Data in these reports which indicate that methylene blue loses its color when milk is drawn anaerobically are not in agreement with numerous observations of ours, except in the case of abnormal udder secretions and milk from some cows going dry.

Summary and Conclusions

A method for exploring variations in milk samples to the action of Staphylococcus and coliform organisms is described. It consists of adding methylene blue to the samples, inoculating them with freshly prepared cultures of the organisms and observing the length of time required to produce a loss of the blue color. Samples from two groups of cows were used. One group contained an average of 12 animals which were fed rations of hay, grain, and silage

or pasture in season. The other group averaged 5 cows, fed hay and grain only.

Variations were observed in the monthly averages of the number of hours needed to produce color changes. The number of hours needed tended to be less during the summers, though not always so. This was particularly noticeable in the month of June in one of the five years. During the same month, a similar lowering of the resistance of milk samples to *Streptococcus agalactiae* was shown, using acid production as the measure. Statistical analysis of the data indicated some of the varying trends in monthly averages were quite significant.

When the cows were first turned out to pasture, a tendency existed in some of the years for the milk samples to temporarily lose their resistance to the Staphylococcus. Oxidation-reduction potentials of the samples collected one year also were noticed to undergo a temporary reduction when cows went to pasture.

Although these tests can not be considered measures of resistance to mastitis, they provide an opportunity to observe environmental factors which, because they apparently influence the test results, provide leads on what may influence the course of the disease.

References

[1] Jackson, C. J.: Factors in the Reduction of Methylene Blue in Milk. J. Dai. Res., *7*, (1936): 31–40.

[2] Mosteller, F.: Note on Application of Runs to Quality Control Charts. Ann. Math. Stat., *12*, (1941): 228–232.

[3] Pounden, W. D.: Variations in the Resistance of Milk to the Activity of *Streptococcus Agalactiae*. Am. J. Vet. Res., *13*, (Oct., 1952): 491–499.

[4] Pounden, W. D., and Frank, N. A.: A Comparison of Increase in Numbers and Acid Production as Measures of Resistance of Milk to *Streptococcus Agalactiae*. Cornell Vet., *4*, (1954): 437–441.

[5] Pounden, W. D., Frank, N. A., Brown, R. W., and Scherer, R. K.: The Resistance of Milk Samples to the Activity of *Streptococcus Agalactiae* as Affected by Feeding Silage. Am. J. Vet. Res., *17*, (April, 1956): 231–234.

[6] Pounden, W. D., Frank, N. A., and Krauss, W. C.: The Resistance of Milk Samples to the Activity of *Streptococcus Agalactiae* as Affected by Pasture. Am. J. Vet. Res., *17*, (April, 1956): 227–230.

[7] Pounden, W. D., Hibbs, J. W., and Edgington, B. H.: The Activity of *Streptococcus Agalactiae* in Milk Possibly Influenced by the Ration. Am. J. Vet. Res., *13*, (Oct., 1952): 486–491.

[8] Pounden, W. D., Pratt, A. D., Frank, N. A., and Smith, H. R.: The Incidence of Mastitis in Cows Fed Legume-Grass Mixtures as Fresh-Cut Crop and as Silage. J.A.V.M.A., *132*, (April 15, 1958): 337–339.

[9] Swed, F. S., and Eisenhart, E.: Tables for Testing Randomness of Grouping in a Sequence of Alternatives. Ann. Math. Stat., *14*, (1943): 66–87.

[10] Wilson, A. T., and Rosenblum, H.: Antistreptococcal Property of Milk. III. The Role of Lactenin in Milk-Borne Epidemics. The *in Vivo* Action of Lactenin. J. Exptl. Med., *95*, (1952): 51–59.

The Morphology and Sporulation of the Oocyst of *Eimeria Brasiliensis*, Torres and Ildefonso Ramos 1939, of Cattle

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AFTER the description of *Eimeria brasiliensis* by Torres and Ildefonso Ramos [8] in 1939, only occasional references were made to this species. Boughton [1] included it as one of the ten valid species of bovine coccidia, and Davis and Bowman [3] stated that it was seen in cattle in Alabama. Rao and Hiregaudar [6] included *E. brasiliensis* among the species of bovine coccidia occurring in India. Lee and Armour [5] compared it, as seen in Nigerian cattle, to the original description.

Eimeria brasiliensis was observed in fecal samples from cattle in Montana in 1954,* and the species has been seen on several additional occasions. It is the purpose of this paper to call attention to this species and to describe its oocysts more completely.

MATERIALS AND METHODS

In October, 1957, 5 newly weaned, crossbred Aberdeen Angus and Hereford calves were obtained from a ranch near Three Forks, Gallatin County, Mont. The calves were housed in a corral at this laboratory, and a total of 194 fecal samples were examined during the fall and winter for the number and species of coccidia. *Eimeria brasiliensis* was identified in eight samples of feces from 2 calves. All the observations reported here were made on the *E. brasilensis* organisms found in these samples; the observations are consistent with less extensive ones made previously here. Length and width measurements were made on 49 specimens, polar cap measurements on 20, sporoplasm measurements on 28, and sporocyst measurements on 9.

Since the number of *E. brasiliensis* appearing in the feces was 56 per gram or less, most observations, photographs, and drawings were made on oocysts concentrated by cover slip flotation in sugar solution. Figure 3 shows a slightly compressed individual photographed in a water-wet mount. Figure 1 shows outlines drawn from photographs. Figure 2 includes only the details which could be seen in the oocyst from which it was drawn.

Oocysts were removed from fecal debris by flotation in sugar solution, and subsequently were washed by centrifugation to remove the sugar. Sporulation trials were carried out in 1 to 2 per cent potassium bichromate at 20 and 30 C.

Oocysts were examined in both bright-field and phase-contrast microscopes with apochromatic objectives. Photographs 3 through 6 were taken in bright-field illumination.

DESCRIPTION OF EIMERIA BRASILIENSIS

Measurements.—The outer shell, including polar cap, measured 37.7 μ (32–40 μ) by 25.1 μ (23–27.5 μ). The wall was 1.5 to 2.0 μ thick, and the length: width ratio was 1.50: 1.0. Fresh sporoplasm measured 26.5 μ or more (up to the complete filling of the oocyst); after 48 hours at 20 C. it was 21.9 μ. Sporocysts were 18.2 μ by 7.9 μ.

Shape.—The oocyst was elliptical, with relatively straight sides curving sharply near the poles. The impression was frequently given that the greatest diameter was closer to the micropyle than the center of the oocyst (fig. 6).

Wall.—The wall of the oocyst was clear and homogeneous. It was of a yellow or greenish appearance in apochromatic objectives and was somewhat thicker at the micropyle end (fig. 4, 5). The micropyle was about 4 μ in diameter (fig. 5).

Polar Cap or Operculum.—The size averaged 7.1 by 1.6 μ (4.0 by 1.5 to 9.0 by 2.3). This structure was colorless and either rounded or nearly flat on top (fig. 2–6), and was frequently somewhat askew (fig. 6). It was seldom absent; only 2 oocysts which lacked the polar cap were identified as *E. brasiliensis*.

Subpolar Body.—The subpolar body ("tenue residual body" of Torres and Ildefonso Ramos [8]) was present in unsporulated specimens and appeared much like the polar cap; it was transparent and colorless. The size and shape varied; the subpolar body was either relatively small and

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reniform (fig. 3), appeared like a cluster of grapes (fig. 5), or was rather diffuse and shapeless (fig. 4). It was frequently lacking or reduced in size in sporulated oocysts. Strands originating in the subpolar body were frequently seen. The oocyst from which figure 2 was drawn showed these strands clearly.

Sporulation.—In the freshly passed oocyst, the sporoplasm filled it laterally and varied in length from 26.5 μ to complete filling of the oocyst. After holding oocysts for 48 hours at 20 C., the sporoplasm was observed to have shrunk to 21.9 μ in diameter. After 120 hours at 20 C., it was 20.0 μ, and after 168 hours, it was 19.2 μ in diameter.

During this process of maturation, before any segmentation was seen, the appearance of the protoplasm changed from a fine, homogeneous granulation (fig. 3, 4) to one that was rather coarse (fig. 5). The granules in the protoplasm appeared to be larger, and the periphery of the sporoplasm had a large number of clear vacuoles, about 3 μ in diameter. There were few or no vacuoles in the center of the protoplasm (fig. 5). Segmentation was first observed after 96 hours at 30 C. and 192 hours at 20 C. Elongation of the sporocysts was first seen after 120 hours at 30 C.

Completion of the process of sporulation was difficult to observe. By the 120th hour at 30 C., the spores had reached their final size, but the ends of the sporocysts were not completely filled with the granular portion of the protoplasm, an indicator of immaturity.

The Sporulated Oocyst.—The sporocysts averaged 18.2 by 7.9 μ in size and had a slight thickening at the more pointed end (fig. 3, 6). Within the sporocyst, lying near its center, was a residual body which measured 4 to 5 μ. The spores themselves were difficult to see in the intact oocyst. In sporocysts which were released through mechanical rupture of the oocyst walls, the spores lay in the ends of the sporocysts. Each spore had, in addition to the nucleus, one to three clear vacuoles. The nucleus itself was inconspicuous, even in phase contrast, and was about 3 μ in diameter. It was distinguished from the vacuoles by its relative obscurity and by granules which lay near the nuclear membrane. The subpolar body was sometimes reduced in size or absent in the sporulated oocyst.

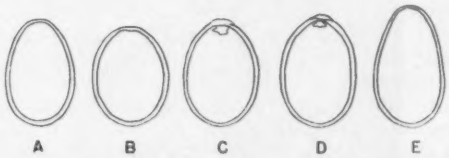

Fig. 1—Relative size and shape of morphologically related oocysts. A and B show E. canadensis; C and D show E. brasiliensis. E shows E. auburnensis.

DIFFERENCES FROM CLOSELY RELATED OOCYSTS

Eimeria bukidnonensis (36.6 by 26.7 μ)[2] averages almost the same size as *E. brasiliensis;* however, the thick, brownish outer wall of *E. bukidnonensis* which tapers sharply toward the micropyle can be clearly seen under low magnification. *Eimeria auburnensis* (38.4 by 23.1 μ)[2] is longer and narrower than *E. brasiliensis,* and gradually tapers toward the micropyle (fig. 1E). In those specimens which do not taper, identification depends on its uniformly thick wall and lack of a polar cap.

The absence of a polar cap and subpolar body is the simplest means of separating *Eimeria canadensis* from *E. brasiliensis.* While *E. canadensis* (32.5 by 23.4 μ)[2] is somewhat smaller than *E. brasiliensis,* their general shapes are similar (fig. 1A–D). The outer shell of *E. canadensis* is thickest at the pole opposite the micropyle, while

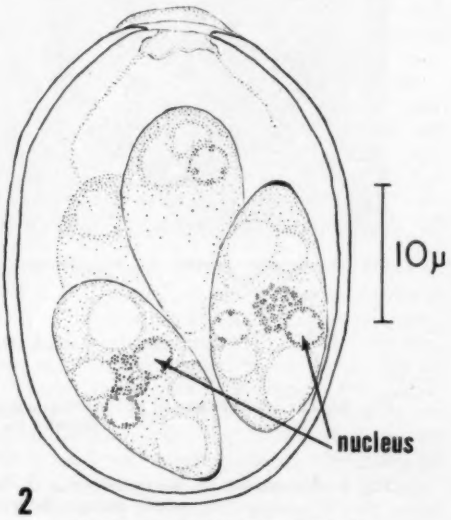

Fig. 2—Sporulated E. brasiliensis.

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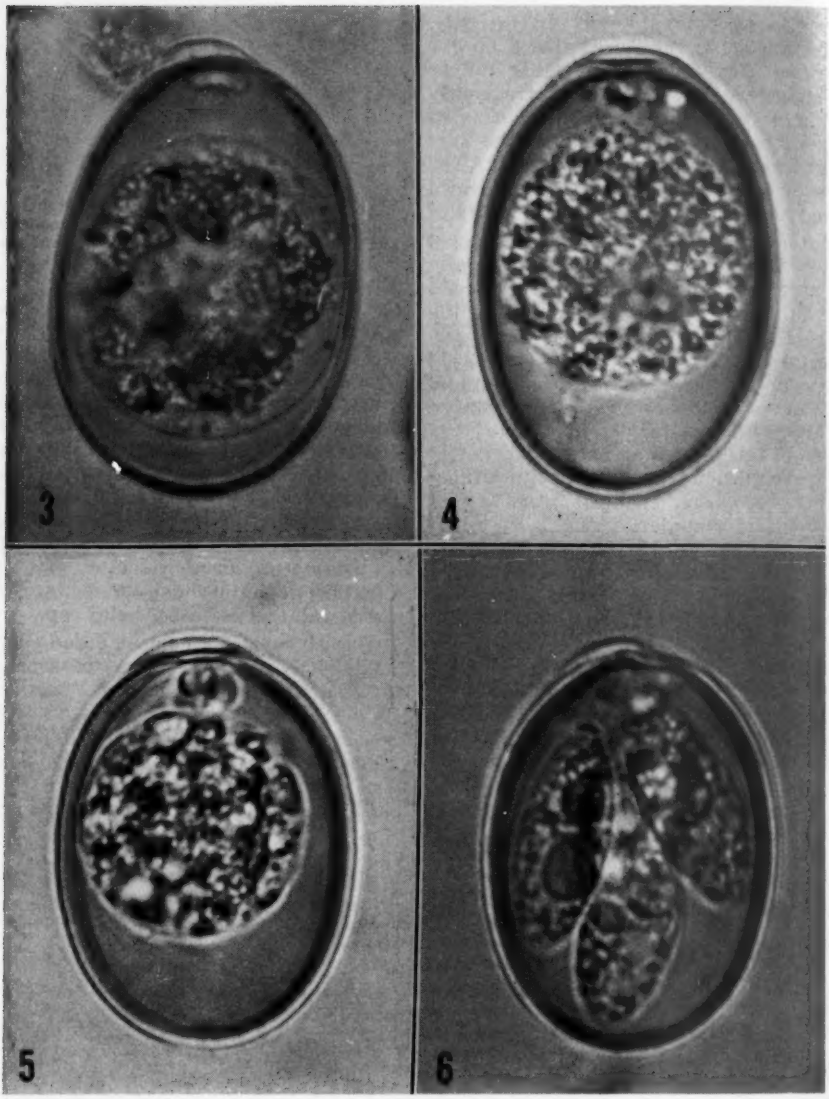

Fig. 3—Freshly passed E. brasiliensis photographed in water-wet mount, and slightly compressed.

Fig. 4—Freshly passed. Notice finely granular protoplasm.

Fig. 5—The sporoplasm, 48 hours after being passed, has become reduced in size and numerous vacuoles lie at its periphery.

Fig. 6—Sporulated. In the sporocyst at the left, the pointed end is up and the slight thickening of the end can be seen. The polar cap is askew.

TABLE 1—Comparison of Descriptions of Eimeria Brasiliensis

Length (μ)		Width (μ)		Sporocyst (μ)									Reference
Av.	Range	Av.	Range	Length	Width	Wall	Color	Shape	Polar cap (μ)	Subpolar body	Micro-pyle	Sporulation time	
37.53	34.20–42.75	27.05	24.22–29.22	.....	....	Smooth	Green to yellow	Ovoid, regular	8.0–10.0 x 2.0–3.0	Present	5–6	144 hours	Torres and Ramos, 1939
38.5*	33.75–49.0	26.5*	24.1–33.2	16.0–21.8	7.2–8.7	.....	Colorless or pink	Oval	10.0–12.0 x 2.0–40	Present	Present	288–336 hours at 27 C.	Supperer, 1952
35.7 ± 0.175	30.7–39.5	25.6 ± 0.134	21.9–29.9	.....	....	.....	Yellow to lavender-pink	Ellipsoidal	1.5–2.0 x 12.0	Present	Present	144–168 hours at 27 C.	Lee and Armour, 1958
37.7	32.0–40.0	25.1	23.0–27.5	18.2	7.9	Clear, homogeneous	Yellow or greenish	Ellipsoidal	7.1 x 1.6	Present	4	120 hours at 30 C. 192 hours at 20 C. for start of segmentation	Present study

* Mode.

the outer shell of *E. brasiliensis* is thickest at the shoulders near the micropyle.

Two species, *Eimeria bombayensis* Rao and Hiregaudar, 1953, and *Eimeria mundaragi* Hiregaudar, 1956, have been reported only from India. *Eimeria bombayensis* measures 37.0 (32.0–40.0) by 22.4 (20.0–25.0) μ, about the same as *E. brasiliensis,* but the micropylar end is pointed, the sides are convex, and there is no polar cap. *Eimeria mundaragi* measures 36.0 to 38.0 by 25.0 to 28.0 μ, has a thin oocyst wall, and is pointed at the micropylar end. Sporulation times for both species are shorter than for *E. brasiliensis.*

Discussion

The oocysts of *E. brasiliensis* found in Montana differ only slightly from those described elsewhere (table 1). The size reported by the original authors was 37.53 (34.20–42.75) by 27.05 (24.22–29.92) μ, while the average in the recent study by Lee and Armour [5] was found to be 35.7 (30.7–39.5) by 25.6 (21.9–29.9) μ. No essential differences, either in structural details or in the relatively long sporulation time, were found between previous descriptions and those reported here.

Supperer [7] described *Eimeria böhmi* from cattle in Austria. The size of *E. böhmi* was given as a mode of 38.5 by 26.5 μ, with a range of 33.75 to 49.0 by 24.1 to 33.2 μ. The presence of a polar cap, the shape and thickness of the wall, and the presence of a sporocyst residual body all are in accord with the description of *E. brasiliensis* (table 1). *Eimeria böhmi* is therefore considered synonymous with *E. brasiliensis.*

Sporulation times are frequently used as criteria for differentiating species of oocysts. *Eimeria brasiliensis* has an extremely long sporulation time and, in my experience, requires longer to begin segmentation than any other coccidium of cattle. Torres and Ildefonso Ramos [8] reported that sporulation required 144 hours, while Supperer [7] reported that it required 288 to 336 hours. In the latter, a temperature of 20 C. was later specified.[5] Sporulation required from 144 to 168 hours at 27 C. in the oocysts from Nigeria.[5] Segmentation was observed in the present work after 92 hours at 30 C. and 192 hours at 20 C., and was complete after 120 hours at 30 C. A reduction in sporulation time by slightly more than half with a 10 C. rise in temperature is consistent with the changes

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in reaction rates seen in other biological systems. The sporulation times reported for *E. brasiliensis* and for *E. böhmi* are consistent with one another, and there is no reason to consider that more than one species is involved.

The occurrence of this species of bovine coccidium in Brazil, India, Austria, Nigeria, and in Alabama and Montana in the United States, indicates a cosmopolitan distribution. Each of the areas has a climate distinctly different from any of the others, and there is therefore reason to believe that *E. brasiliensis* will be found wherever cattle are raised.

SUMMARY

1) *Eimeria brasiliensis* Torres and Ildefonso Ramos, 1939, of cattle was reported from Montana, and the structure and sporulation of its oocyst compared to the original description.

2) *Eimeria böhmi* Supperer, 1952, is considered synonymous with *E. brasiliensis.*

3) *Eimeria brasiliensis* has been reported in Brazil, India, Austria, Nigeria, and in Alabama and Montana in the United States. It would appear that this species has world-wide distribution.

References

[1] Boughton, D. C.: Bovine Coccidiosis: From Carrier to Clinical Case. N. Am. Vet., *26*, (1945): 147–153.

[2] Christensen, J. F.: The Oocysts of Coccidia from Domestic Cattle in Alabama (U.S.A.) with Descriptions of Two New Species. J. Parasitol., *27*, (1941): 203–220.

[3] Davis, L. R., and Bowman, G. W.: Coccidiosis in Cattle. Proc. U.S. Livestock San. A., 55th Ann. Meeting (1951): 39–50.

[4] Hiregaudar, L. S.: A New Species of Eimeria from a Cow-Calf in Bombay State. Cur. Sci., *25*, (1956): 197.

[5] Lee, R. P., and Armour, J.: A Note on *Eimeria Braziliensis* Torres and Ramos 1939, and Its Relationship to *Eimeria Böhmi* Supperer 1952. J. Parasitol., *44*, (1958): 302–304.

[6] Rao, S. R., and Hiregaudar, L. S.: Coccidial Fauna of Cattle in Bombay State, with Particular Reference to a Recent Outbreak at Aarey Milk Colony Together with a Description of Two New Species, *E. Bombayensis* and *E. Khurodensis*. Bombay Vet. Coll. Mag., *4*, (1954): 24–28.

[7] Supperer, R. Die Coccidien des Rindes in Österreich. Österr. zoologische Ztschr., *3*, (1952): 591–601.

[8] Torres, S., and Ildefonso Ramos, J.: Eimeria dos Bovinos em Pernambuco. Arq. Inst. Pesquisas Agron., *2*, (1939): 79–96.

Evaluation of the Hemolytic Test in Canine and Bovine Leptospirosis

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A PREVIOUS REPORT from this laboratory[1] described a hemolytic (HL) reaction which involved the ability of leptospiral extracts to sensitize sheep erythrocytes to hemolysis in the presence of anti-leptospiral serum and complement. Subsequent reports have described the preparation of HL antigen from *Leptospira biflexa*,[2] the standardization and stabilization of the antigen and its use in the HL test,[2] and have evaluated the use of the HL test in human leptospirosis.[3]

Recently, the HL antigen has been serologically investigated.[4] The evaluation studies demonstrated generic specificity of the HL antigen, as well as its marked sensitivity with rabbit and human antiserums and suggested that currently used microscopic agglutination (MA) tests could be advantageously supplanted by the HL procedure in the serodiagnosis of human leptospirosis.

As a result, some interest has been generated concerning the possible application of the HL test to various animal serums, especially bovine and canine serums. Although the author does not personally have access to such serums, various colleagues have supplied canine and bovine serums from a relatively small number of experimentally infected animals and a fairly large number of randomly selected bovine serums. This report describes comparative HL and MA titrations on these serums.

MATERIALS AND METHODS

Unless otherwise stated, MA tests were performed in this laboratory following the method of Gochenour,[5] except that serums were decimally diluted. The HL tests were performed on canine serums as previously described for use with human serums;[2] however, initial attempts to apply the HL test to bovine leptospirosis yielded titers which were disappointingly low in comparison with MA titers.

Considerable increase in sensitivity was achieved by the addition of 1 per cent (final concentration) human plasma fraction V to the veronal used as complement diluent. Some additional increase in sensitivity was obtained by using 128 units of HL antigen in place of the 16 used for human serums. Bovine serum albumin was less active than human plasma fraction V in enhancing sensitivity with bovine serum.

The use of these two modifications gave HL sensitivity with bovine serums approaching that obtained with human serums. Therefore, all bovine serums were HL-tested using the originally described HL procedure,[2] except for the incorporation of human plasma fraction V in the complement diluent and the increased amount of antigen.

PROCEDURE AND RESULTS

The HL and MA titrations were compared on serums from 13 dogs experimentally infected with *Leptospira icterohaemorrhagiae*.* Available serum samples were collected before infection and at weekly intervals for three weeks after infection. The average MA and HL titers reached a peak at comparable levels at the end of the first week after infection, but HL titers seemed to decline at a faster rate thereafter (table 1).

TABLE 1—Microscopic Agglutination (MA) and Hemolytic (HL) Antibody Responses of 13 Dogs Experimentally Infected with Leptospira Icterohaemorrhagiae

	Titer reciprocals			
	MA		HL	
Days after infection	Average	Geometric mean	Average	Geometric mean
0	<25	.....	<10	.....
7	9,300	5,000	10,350	3,200
14	9,200	5,600	2,400	1,000
21	5,200	3,400	1,800	800

Comparative HL and MA titrations were performed on serums from 8 calves† experimentally infected with *Leptospira sejroe*. From average comparative titers (table 2), it may be seen that a peak occurred in the MA titers on the eleventh to sixteenth

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* These serums were made available and MA titrations were performed by L. C. Murphy and A. D. Alexander, Division of Veterinary Bacteriology, Walter Reed Army Institute of Research, Washington, D.C.

† These serums were made available and MA titrations were performed using homologous antigen by Mildred Galton, CDC Laboratories, Chamblee, Ga.

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day and in the HL titers on the eleventh day. However, the average HL titers decreased more rapidly after reaching their peak than did MA titers.

The distribution of HL titers by MA titers on 653 randomly selected bovine serums** is presented (table 3). Most of these animals came from central South Dakota. The MA test was performed with *Leptospira pomona* antigen. The percentage of total serums showing MA and HL reactions at different levels of sensitivity show good agreement. It is interesting to calculate from this table that 17.5 per cent (114) of the serums showed titers of less than 1:10 by both tests, and slightly over 59.0 per cent (386) showed reactions of 1:10 or higher to both tests, making an additive general agreement of 76.6 per cent. Those serums showing disagreement represented 23.4 per cent (153), of which 11.6 per cent (76) had HL titers of 1:10 or higher and MA titers of less than 1:10, and 11.8 per cent (77) had HL titers of less than 1:10 and MA titers of 1:10 or higher. Thus, these serums were about equally divided with respect to reactivity with MA or HL testing procedures.

** These serums were made available by W. F. Waddell, federal veterinarian in Pierre, S. Dak.

TABLE 2—Microscopic Agglutination (MA) and Hemolytic (HL) Antibody Response in 8 Calves Experimentally Infected with Leptospira Sejroe

Days after infection	No. of serums	Titer reciprocals: MA Average	MA Geometric mean	HL Average	HL Geometric mean
0–4	15	<16	.....	<10	.....
6–8	7	430	160	390	200
11	5	2,460	1,600	10,600	2,500
12–13	7	3,080	1,000	2,030	720
14–16	6	3,670	1,600	700	400
18–23	8	1,220	800	540	200
27–33	10	170	135	150	20
35–47	8	104	250	47	21

Discussion

The HL titers reached maximum levels as high as MA titers in the serums from experimentally infected dogs and calves, but HL titers decreased at a more rapid rate. Although individual serum titrations are not shown, in neither group of animals was this more rapid decrease due to HL-anergic animals. Rather, the average decrease reflected general decrease by individual serums. This more rapid decrease in HL titers is contrary to previous comparative HL and MA titrations in human leptospirosis,[3] and may account in part for the 23 per cent disagreement in randomly selected bovine serums.

The HL and MA titers showed fairly close agreement (77%) with 653 randomly selected bovine serums. Those showing disagreement (23%) were about equally divided with respect to reactivity with each test. Those which were MA-reactive and HL-nonreactive could indicate infections in the more distant past, since evidence is presented for the more rapid decrease in

TABLE 3—Distribution of Hemolytic (HL) Titers by Microscopic Agglutination (MA) Titers on Randomly Selected Bovine Serums

MA titer (reciprocals)	Distribution by HL titers (reciprocals) in numbers: <10	10	100	1,000	10,000 or higher	Serums (No.)	Serums (%)
<10	114	28	34	9	5	190	29
10	22	17	24	7	0	70	11
100	18	19	26	10	2	75	12
1,000	17	14	33	30	27	121	18
10,000 or higher	20	16	49	75	37	197	30
Total No. of serums	191	94	166	131	71	653	....
Per cent of total	29	14	26	20	11	.....	....

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HL titers in calves and dogs. Those which were HL-reactive and MA-nonreactive could indicate infections with serotypes other than *L. pomona* (the MA testing antigen), since the MA reaction is type-specific.[6]

It has been previously reported that it is necessary to employ a battery of 17 or more different antigens in order to insure detection of agglutinins that may be provoked by 60 or more different leptospiral serotypes.[3] The HL antigen has been previously shown to be genus-specific when tested against rabbit and human antibodies,[3] and additional evidence is here presented in the reactivity of HL antigen with canine antibodies against *L. icterohaemorrhagiae* and with bovine antibodies against *L. sejroe* and probably *L. pomona*.

Final resolution of the significance and clinical importance of HL titers in canine and bovine serums must obviously await more extensive studies; however, present studies on a relatively small number of animals would seem to indicate potential usefulness of the HL procedure in canine and bovine leptospirosis and to warrant more extensive investigations by others.

SUMMARY

Hemolytic (HL) and microscopic agglutination (MA) antibody titers reached peaks at comparable levels and times in serums from 13 dogs experimentally infected with *Leptospira icterohaemorrhagiae* and 8 calves experimentally infected with *Leptospira sejroe*. However, HL titers decreased at more rapid rates than MA titers. Comparative HL and MA titrations on 653 bovine serums showed 77 per cent general agreement. Possible significance of these findings is discussed.

References

[1] Cox, C. D.: Hemolysis of Sheep Erythrocytes Sensitized with Leptospiral Extracts. Proc. Soc. Exptl. Biol. & Med., *90*, (1955): 610–615.

[2] Cox, C. D.: Standardization and Stabilization of an Extract from *Leptospira Bifleza* and Its Use in the Hemolytic Test for Leptospirosis. J. Infect Dis., *101*, (1957): 203–209.

[3] Cox, C. D., Alexander, A. D., and Murphy, L. C.: Evaluation of the Hemolytic Test in Human Leptospirosis. J. Infect. Dis., *101*, (1957): 210–218.

[4] Cox, C. D., Stover, R. C., and Treick, R. W.: Serological Studies on Hemolytic Antigen from Leptospira. Proc. Soc. Exptl. Biol. & Med., *98*, (1958): 265–269.

[5] Gochenour, W. S.: Symposium on the Leptospiroses. Army Medical Service Graduate School, Walter Reed Medical Center. (1953): 160.

[6] Wolff, J. W.: The Laboratory Diagnosis of Leptospirosis. Charles C Thomas, Springfield, Ill., 1954. 1954.

Colonic Intussusception in the Mouse

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IN CONNECTION with a radiation-genetics project with mice at this laboratory, 14 mice with cases of fatal colonic intussusception have been encountered. Since intussusception of any sort has apparently never previously been reported in this species *(Mus musculus)*,[2,5] the circumstances may be of general interest.

HISTORY AND CLINICAL SIGNS

The first case was found Dec. 19, 1955. The mouse was a mature (6 months old) black-and-tan male of crossbred origin. Previously he had been healthy and vigorous, but on this date he showed cachexia, with a slight bloody exudate from the anus. The animal was therefore killed for necropsy. The only abnormal condition found was intussusception, the anterior portion of the colon having been drawn into the posterior.

During the ensuing three years, 13 additional mice with colonic intussusception have been found, all descendants of this male and of his sibs. The origins and relationships of these mice are outlined in the pedigree chart (fig. 1). More cases no doubt escaped recognition, since examinations were not made daily and decomposing specimens were not dissected.

Of the 14 mice with recognized cases, 6 were males, varying in age from 1 month (weaning age) to 1 year. The females were all mature, and several were nursing litters. None was visibly pregnant. In 1 female which died (14 months old), the intussusception was included in an extensive rectal-colonic prolapse.

In general, the clinical signs were the same as in the first case, and death seemed imminent. Several cases were found after death. Since there was considerable inflammation of the affected colon and strangulation of the included blood vessels, the mice probably died within a few days after signs appeared. Judging from the appearance at necropsy, one can infer that the invagination probably began in the middle region of the colon, and proceeded to draw the anterior portion in until the cecum was reached (fig. 2) which, in the mouse, was large enough to block further movement. Apparently the intussusception completely prevented fecal passage. In some cases, the cecum and small intestine were considerably distended, but the cachexia resulted in reduced food intake.

This family of mice originated from crossing several stocks maintained in this laboratory, namely "S," "P," "yellow," and "belt." The primary object was the analysis and maintenance of a color mutation *(a^e)* obtained in an x-ray experiment.[3] None of the mice with intussusception had been irradiated, but all had this mutant gene, either homozygous or heterozygous. The association is probably coincidental, since hundreds of genetically comparable mice in this family have lived to old age without this pathological development. The total observed frequency of intussusception has been less than 3 per cent in this family, and zero in all the other stocks.

DISCUSSION

Occasionally, among various genetically different stocks in this laboratory there have been mice with chronic prolapsus ani. This has occurred about equally in both sexes, almost always in mature or old animals. Whether it is in any way related to the colonic intussusception is unknown. The limitation of intussusception to a single family line suggests that a unique factor is involved.

Intestinal intussusception has often been reported in a number of kinds of mammals, including rabbits,[5] dogs,[6] ungulates,[7] and man.[1] Various locations have been involved, but the colon much more rarely than the small intestine. Precipitating factors have been diverse—tumor, parasites, some other obstructing foreign body, inflammation, or violent peristaltic movement following some sort of shock, such as drinking very cold water. However, often no inciting

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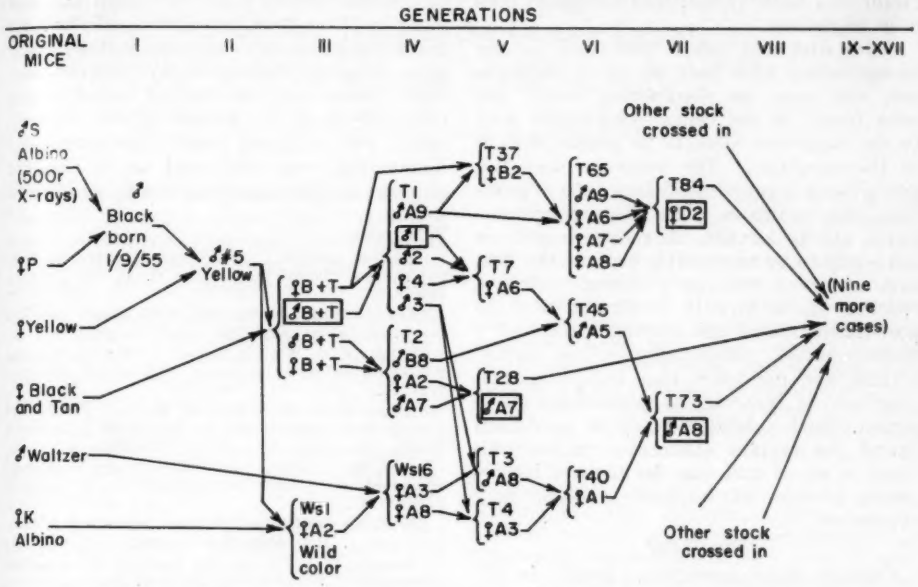

Fig. 1—Pedigree chart for the family of mice in which intussusception appeared. Numbers without sex identification are mating numbers. Designations for the 5 first-affected mice are enclosed in rectangles. Occurrence in generations IX–XVII was less frequent, so details of relationship are not shown.

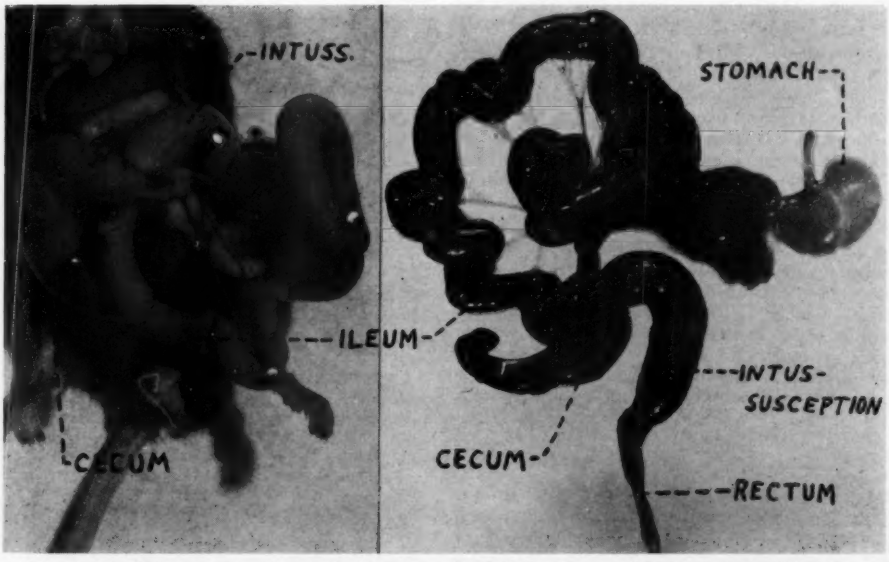

Fig. 2—Typical examples of colonic intussusception: left—from a female (preserved in formalin), showing distended cecum; right—from a male which died (fresh specimen).

factor has been found, and idiopathy has been suggested.

Since diet and other conditions in our mouse colony have been standard throughout, and since no obstructing factor has been found in the colon, inheritance may be an important element in predisposition to the condition. The evidence does not permit an adequate mendelian type of analysis but indicates that the inheritance could not be simple. A recessive gene is not likely to be responsible because the first case observed was in a direct outcross. Neither can a strictly dominant gene be postulated, unless the crossing produced a disharmonious combination. The rather erratic age incidence also indicates complexities of genetic-environmental interaction. Such subtleties of cause have been found for certain other abnormal conditions in mice [4] and may be of more importance in medicine than is commonly recognized.

SUMMARY

Colonic intussusception, found in 14 crossbred mice of various ages, was limited to a single family line. Although affected mice had not been irradiated, all had an homozygous or heterozygous mutant color gene obtained through x-ray experimentation. Intussusception was not found in any other stocks in the mouse colony. Inheritance, with complex genetic-environmental interaction, was indicated as a possible element in predisposition to this condition.

References

[1] Editorial reviews: Lancet, *264*, (1953): 229–230; Lancet, *269*, (1955): 145; J. Am. M. A., *159*, (1955): 153.

[2] Grüneberg, H.: The Genetics of the Mouse. Bibliogr. Genet., *15*, (1952): 372–374.

[3] Hollander, W. F., and Gowen, J. W.: An Extreme Non-Agouti Mutant in the Mouse. J. Hered., *47*, (1956): 221–224.

[4] Hollander, W. F., Gowen, J. W., and Stadler, J.: A Study of 25 Gynandromorphic Mice of the Bagg Albino Strain. Anat. Rec., *124*, (1956): 223–243.

[5] Jaffé, R.: Anatomie und Pathologie der Spontanerkrankungen der kleinen Laboratoriumstiere. J. Springer, Berlin, 1931.

[6] Joest, E.: Spezielle pathologische Anatomie der Haustiere. vol. 1. R. Schoetz, Berlin, 1919.

[7] Nieberle, K., and Cohrs, P.: Lehrbuch der speziellen pathologischen Anatomie der Haustiere. 3rd ed. Gustav Fischer, Jena, 1949.

Study of the Sperm Capacitation Factor in the Genital Tract of the Female Rabbit

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IT HAS BEEN SHOWN [2] that the presence of the male accessory genital gland secretion interferes with the fertilization of the ovocytes and the development of the embryo. However, neither epididymal spermatozoa nor dilute semen has any untoward effect on fertilization and, if these are deposited in the oviduct or uterus of the rabbit from three to four hours after mating (6 or 7 hours prior to ovulation), as a rule fertilization of the ova will take place.[1,3] However, if the deposition of semen happens later than four hours before ovulation, no fertilization occurs.

The apparent need for the spermatozoon to spend a certain amount of time in the oviduct or uterus before a successful fertilization can take place indicates the presence of a factor in the higher segments of the female genital tract whose conditioning or capacitating activity on the sperm is an apparent necessity before a successful fertilization can take place. For want of a better term, this factor has been called the "capacitation" factor.

A series of experiments has been devised to establish whether normally ejaculated spermatozoa which are free of accessory genital gland secretion after deposition into the higher reaches of the female genital tract are *(a)* capable of fertilizing an ovum, or *(b)* whether the activity of the capacitation factor is needed in this case as well, to complete fertilization.

MATERIALS AND METHODS

Sexually mature Dutch rabbits were used as the experimental animals. The females were kept in separate cages within the animal house for at least three weeks preceding the date of the experiment to assure the absence of pseudopregnancy. A number of males were trained to ejaculate into an artificial vagina. Other male rabbits were vasectomized.

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Since it is known that ovulation in the rabbit takes place ten hours after mating, doe rabbits were mated with vasectomized bucks (referred to as a "sterile mating") then held for the required lapse of time before insemination.

For insemination, with the exception of the control experiments, once- or twice-washed rabbits' spermatozoa were used. The procedure was as follows: Semen was obtained with the help of an artificial vagina, which is a modification of Macirone-Walton's[4] old model. Immediately after ejaculation, the semen was tested for sperm motility and, if the motility was satisfactory, the semen was mixed with an equal amount of citrate buffer (pH 7) and centrifuged with a relative centrifugation force of 5,000 to 10,000 (R.C.F. = $284 \times 10^{-7} \times R \times N^2$). If the centrifugation force was greater, the spermatozoa were found to fracture. At lower values, the spermatozoa did not separate well enough from the other constituents of the semen.

After the first centrifugation, the supernatant fluid was pipetted off; the separated spermatozoa were then mixed with a new addition of citrate buffer and either used in this once-washed condition or centrifuged once more. After the second centrifugation, the supernatant fluid was discarded again and the sperm motility tested after the addition of fresh citrate buffer. If the spermatozoa still showed adequate motility, they were used in the experiments.

Laparotomy was performed under aseptic conditions on pentobarbital-anesthetized rabbit does, and the animals were inseminated in both ampullae of the fallopian tubes or in the middle third of both uterine horns. The ovaries were scanned for the presence of graafian or newly ruptured follicles.

Some of the does were killed within six to ten hours after mating in an attempt to observe sperm penetration and the union of pronuclei. However, the absence of phase-contrast equipment made the observation difficult, so they were killed a week after insemination when the blastocyst formation was well advanced. Experiments were considered positive where well-developed blastocysts were obtained.

EXPERIMENTAL PROCEDURE

Control Series.—A control experiment was performed on 4 does. Each of them was inseminated by placing the citrate washing of epididymis spermatozoa into the ampullae of both fallopian tubes seven

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hours prior to ovulation. Both became fertilized, thus repeating Austin's [1] results.

Experiment 1.—For the first experiment, normally ejaculated semen was used. Nine does were inseminated in the ampullae of the fallopian tubes six hours after sterile mating.

A total of 54 ovocytes and blastocysts were recovered. Of the total there were 23 atrophic unfertilized eggs; ten atrophic fertilized eggs; 11 fragmented eggs; two atrophic morulae; and eight atrophic blastocysts (table 1).

Experiment 2.—Five does were inseminated in both ampullae with once-washed spermatozoa. Of the 23 ovocytes recovered from the 5 does one week later, 16 were fertilized but degenerating, two were atrophic morulae, and five were atrophic blastocysts (table 1).

Experiment 3.—Six hours after sterile mating, twice-washed spermatozoa were deposited in the ampullae of 6 does. A total of 18 apparently normal blastocysts and five atrophic ova were recovered after seven days (table 1).

Experiment 4.—Six hours after sterile mating, twice-washed spermatozoa were deposited in the middle third of both uterine horns in each of 3 does. Fourteen apparently normal blastocysts were recovered (table 1) one week later from 2 does and only degenerated ones from the third doe.

Experiment 5.—The aim was to deposit twice-washed spermatozoa in the oviducts of 6 does two hours after ovulation (12 hours after mating). Seven days after the operation, 21 blastocysts were recovered from 5 does and only atrophic eggs from 1 animal. The blastocysts obtained in this experiment were much smaller than those in the previous experiment, and some of them were in a collapsed state (table 1).

DISCUSSION AND CONCLUSIONS

These experiments have shown that two important factors must be considered to achieve successful fertilization with ejaculated semen deposited in the upper portions of the female genital tract: (1) the degree of washing, and (2) the time interval between mating and insemination or, rather, between insemination and ovulation.

Insemination into the oviduct with whole semen never resulted in fertilization, and insemination with once-washed sperm never

TABLE 1—The Number of Experiments, Animals Used, Number of Ova Recovered, and Their Developmental Stages or Other Conditions

Experiment No.	No. of animals	Unfertilized atrophic eggs	Fertilized atrophic eggs	Fragmented vitelline material	Atrophic morulae	Atrophic blastocysts	Normal blastocysts	Smaller than normal blastocysts	Corpora lutea	Blood follicles	Ovocytes
1	1	4	..	1	..	..	..	..	6	..	5
	2	5	..	..	..	..	..	..	6	3	5
	3	6	..	..	..	..	..	..	6	..	6
	4	4	..	..	2	..	..	..	7	..	6
	5	..	..	6	..	..	..	..	6	2	6
	6	4	..	..	..	2	..	..	6	..	6
	7	..	1	4	..	3	..	..	8	..	8
	8	..	1	..	..	3	..	..	11	2	4
	9	..	8	..	..	..	..	..	10	..	8
2	1	..	3	..	..	1	..	..	8	..	4
	2	..	3	..	..	3	..	..	9	..	6
	3	..	3	..	..	..	..	..	7	..	3
	4	..	3	..	2	..	..	..	9	..	5
	5	..	4	..	..	1	..	..	7	..	5
3	1	..	..	..	..	..	5	..	9	..	5
	2	..	2	..	..	..	2	..	4	..	4
	3	..	2	..	..	..	2	..	4	..	4
	4	..	..	..	..	..	4	..	7	..	4
	5	..	1	..	..	..	3	..	7	..	4
	6	..	..	..	..	..	2	..	5	..	2
4	1	..	4	..	..	..	5	..	9	..	9
	2	..	..	..	..	..	9	..	9	..	9
	3	..	1	..	..	3	..	..	6	2	4
5	1	8	..	..	..	..	..	2	10	..	10
	2	4	..	..	..	..	..	3	8	..	7
	3	..	..	..	..	..	..	4	4	4	4
	4	..	..	..	..	..	..	9	9	..	9
	5	6	..	..	..	..	..	..	10	..	6
	6	..	..	..	..	..	..	3	3	..	3

resulted in blastocyst formation, although cleavage and early embryological development apparently took place. The only successful experiments were those in which twice-washed spermatozoa were used. Therefore, for successful insemination into the higher segments of the genital tract, thorough washing appears to be extremely important.

Concerning the time limits between which successful insemination can take place, it can be assumed that the earliest period would be the time of mating. Successful insemination was achieved six hours after mating, but not two hours after ovulation. Since the ovum of the rabbit can still be fertilized two hours after ovulation, the failure of this to occur is ascribed to the lack of capacitation of the sperm.

SUMMARY

A number of experiments were conducted on rabbits to determine whether normally ejaculated semen can be used for insemination into the uterus and oviduct and, if so, what its relationship is to mating and ovulation.

1) Twice-washed spermatozoa were found to fertilize rabbit ova and cause early embryological development. Once-washed spermatozoa apparently fertilized ova and induced cleavage, but the embryos degenerated in early blastocyst stage. Normal ejaculatum did not fertilize any ovum.

2) Fertilization was not successful two hours after ovulation (12 hours after mating) but it was successful four and six hours after mating (6 to 4 hours prior to ovulation), showing an apparent need for "capacitation."

References

[1] Austin, C. R.: The "Capacitation" of the Mammalian Sperm. Nature, London, *170*, (1952): 326.

[2] Chang, M. C.: The Effect of Seminal Plasma on Fertilized Rabbit Ova. Proc. U.S. Nat. Acad. Sci., *36*, (1950): 188–191.

[3] Chang, M. C.: Fertility and Sterility as Revealed in the Study of Fertilization and Development of Rabbits' Eggs. Fert. & Ster., *2*, (1951): 205–222.

[4] Macirone, C., and Walton, A.: Fecundity of Male Rabbits as Determined by "Dummy Matings." J. Agric. Sci., *28*, (1938): 122–134.

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The Effect of Antibiotics on Semen Production in Rabbits

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THE OPINION is prevalent that fertility is often low in farm animals following an acute illness, the recovery from which has been accelerated by treatment with antibiotics. The question naturally arises as to whether the fever which developed with the illness or the treatment with antibiotics caused the impairment in fertility. Some producers feel that the antibiotics contribute to the lowered fertility, but little information on the subject is available. Antibiotics are also used in the treatment of certain diseases after which fertility is markedly improved. It seemed important to determine what influence antibiotics at therapeutic doses have on fertility in animals that are not suffering from any known illness. Male rabbits were used for the study because they are inexpensive to maintain and semen can be readily collected from them for laboratory study.

REVIEW OF LITERATURE

There have been few papers directly bearing on the effects of antibiotics on semen production. Some yield information on restricted aspects of reproduction in the male. For example, the feeding of antibiotics had neither a detrimental nor beneficial effect on the percentage of conception, number of live pigs farrowed per litter, birth weight of pigs, or weaning weight.[1-4] This implies no residual effect of antibiotics in the male.

Rusoff *et al.*,[5] on adding a chlortetracycline (Aureomycin*) supplement to the ration (level in the grain mixture equivalent to intake of approximately 300 mg. of chlortetracycline daily per animal), found no alteration in the number of ejaculations, volume per ejaculation, sperm per milliliter, sperm per ejaculation, and the nonreturn rate of bulls.

Uram *et al.*[11] found that when groups of 10 male and 30 female weanling rats were fed one of three diets (basal, basal plus 0.04% oxytetracycline [Terramycin†], or basal plus 0.04% streptomycin), there was no significant difference in reproductive ability among the groups of rats fed the three diets.

Sciortino [6] noticed only slight histological changes in the testicular tissue of 4 rabbits treated with 3,600,000 to 7,690,000 units of penicillin.

Thus, the literature provides little evidence directly or indirectly on the effect of antibiotics on semen production generally or in the rabbit in particular.

EXPERIMENTAL PROCEDURE

It was the purpose of this experiment to study the effect of intramuscular injections of antibiotics at therapeutic levels on semen production in the rabbit. The antibiotics were given intramuscularly to prevent alimentary destruction or failure of absorption. The therapeutic level used was 10 times greater than the amounts fed in nutritional studies. The properties of the semen to be studied were the volume per ejaculation, motility, percentage of live sperm, concentration, survival during storage, and morphology of the spermatozoa.

Experimental Animals.—New Zealand White male rabbits, 5 months old, were randomly allotted to four groups of 3 each. Animals in the first three groups were treated with antibiotics (oxytetracycline, penicillin, and streptomycin) and the fourth group served as controls. All animals were fed commercial rabbit pellets. Each rabbit was kept in a separate wire cage and adequate feed and water were given each morning to supply it for the day.

Each antibiotic was injected as an aqueous solution intramuscularly daily for three weeks, as follows: group 1, oxytetracycline, 2 mg. per kilogram of body weight; group 2, penicillin, 4,000 units per kilogram of body weight; group 3, streptomycin, 40 mg. per kilogram of body weight; and group 4, control group, no antibiotics. During the treatment, semen samples were taken once a week to study changes that might occur. The study was continued for five weeks subsequent to antibiotic treatment. The injections were started June 1, 1957.

Treatment of Semen.—The semen was collected once a week for two months by use of an artificial vagina, designed by Lambert and McKenzie.[6] The volume was calculated by measuring in millimeters the height of a column of semen in calibrated vials. General appearance, such as the color, viscosity, and opacity, was also recorded.

Technical paper No. 1176, from the Oregon Agricultural Experiment Station, Oregon State College, Corvallis. This study was conducted in cooperation with the Agricultural Research Service, and State Experiment Stations under Western Regional Project W-1 on Beef Cattle Breeding Research and Western Regional Project W-46 on the Effects of Environment Stresses on Range Cattle and Sheep Production.

* Aureomycin is produced by Lederle Laboratories, Division of American Cyanamid Co., New York, N.Y.

† Terramycin, produced by Pfizer Laboratories, Division of Chas. Pfizer & Co., Inc., Brooklyn, N.Y.

Concentration of spermatozoa, expressed in numbers per cubic millimeter, was determined by use of a Spencer hemacytometer.† By observing semen under the microscope, motility was rated 10 to 0, with 10 denoting maximum motility.[7, 12] The percentage of live spermatozoa was determined from smears stained with fast green eosin.[9] On this same slide, the percentage of abnormal spermatozoa was determined by counting the number of abnormal cells present in a total of 300 spermatozoa. Spermatozoa considered abnormal were those without tails, with broken or looped tails, headless, abnormally large-headed, small-headed, two-headed, having a pyriform (pear shaped) head, and those with enlarged middle pieces.

Survival Time During Storage.—The semen samples for each collection were diluted with four volumes of heated homogenized milk as an extender[10] and were stored in the refrigerator at 40 to 44 F. Each sample was rated for motility at 48-hour intervals until motility was zero; thus, a time limit of useful storage was established.

RESULTS

For clarity and convenience, the observations are presented as follows: semen volume, initial motility, sperm concentration, percentage of live and abnormal spermatozoa, and survival time during storage.

Semen Volume.—Average (calculated from 3 rabbits) volume of semen per ejaculation varied from 0.86 to 1.33 ml. among the four groups during the control period. Average semen volume during the treatment period varied from 0.82 to 1.14 ml. for the treated groups and was 0.9 ml. for the control group. During the post-treatment period, average volume for the treated groups varied from 0.78 to 1.35 ml. per ejaculation, while the average volume of semen from control rabbits during this period was 0.87 ml. Evidence of an alteration in semen volume due to oxytetracycline, penicillin, and streptomycin was not obtained (table 1).

Sperm Concentration.—Average values for the concentration of spermatozoa in semen varied from 559 to 684 million per milliliter over the four groups during the two month control period (table 1). Usually low values of semen concentration were irregularly obtained after treatment with oxytetracycline and streptomycin, and in the control rabbits. A statistically significant effect of the antibiotics on sperm concentration was not established. If there is such an effect, it is variable from rabbit to rabbit and from time to time in the same rabbit, with both unusually high and unusually low values of spermatozoan concentration being obtained.

Total Number of Spermatozoa per Ejaculation.—The average number of spermatozoa for the four groups during the control period varied from 548 to 746 million per ejaculation. The data suggest that prolonged administration of oxytetracycline may lead to a reduction in the number of spermatozoa per ejaculation. Cessation of oxytetracycline administration was followed by an increase in the number per ejaculation. Effects of pencillin and streptomycin, if any, were evanescent (table 1).

Spermatozoan Motility.—Spermatozoan motility varied from group to group, and from time to time in the control period (table 1). Spermatozoan motility in semen, as collected, was somewhat higher during treatment in the groups given antibiotics than it was during the control period or in the control group. Spermatozoan motil-

† Spencer hemacytometer, manufactured by the American Optical Co., Buffalo, N.Y.

TABLE 1—The Effect of Antibiotics on Semen Production in Rabbits

Group	Collection	Average					
		Volume (ml.)	Conc./cmm. (thousands)	Total No. (millions)	Motility (0–10)	Alive (%)	Abnormal (%)
1. Oxytetracycline (2 mg./kg. daily) i.m.—21 days	Before treat.	1.33	559	746	6.83	89	16
	During treat.	1.14	557	635	7.32	88	12
	After treat.	1.35	587	795	7.86	89	13
2. Penicillin (4,000 units/kg.) i.m.—21 days	Before treat.	0.86	632	548	7.07	84	16
	During treat.	0.82	871	714	7.99	94	17
	After treat.	0.78	774	608	7.86	93	12
3. Streptomycin (40 mg./kg. daily) i.m.—21 days	Before treat.	1.01	684	693	7.26	87	18
	During treat.	0.95	617	586	7.65	92	11
	After treat.	0.87	819	715	8.06	92	13
4. Control	Before treat.	0.99	605	600	7.37	88	20
	During treat.	0.90	484	436	6.88	91	11
	After treat.	0.87	723	631	7.93	88	12

i.m. = intramuscularly.

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ity was higher after the cessation of treatment with all three antibiotics than during antibiotic treatment.

Percentage of Live Sperm in Fresh Semen.—There were no clear-cut directions of change in percentages of live sperm in fresh semen in either treated or control groups (table 1). There may be some indication of an increase in the percentage of live sperm during and after treatment with penicillin.

Percentage of Abnormal Sperm.—The average percentage of abnormal sperm in fresh semen (table 1) varied from 16 to 20 among the groups during the control period. In the control groups, the average percentage of abnormal sperm fluctuated from time to time and ranged between 11 and 20 per cent. No clear-cut effects of the antibiotics on the percentage of abnormal sperm were established. Although the percentage of abnormal sperm decreased during and following treatment in the groups receiving antibiotics, these decreases appeared to be under other influences because the same decreases in abnormal sperm occurred in the control group during these periods.

Sperm Survival Time During Storage.—The time limit for successful storage of semen samples was determined by the examination of motility of the spermatozoa in diluted semen. The storage limit was not altered significantly by the administration of oxytetracycline, penicillin, or streptomycin (table 2). Data from both control and treated rabbits showed an unexpected, unexplained, and roughly parallel increase in magnitude with time.

There was much variation in all semen characteristics studied both between rabbits in different groups and between collections from the same rabbit. The differences among animals in treated groups or between any treated group and the controls were not of sufficient magnitude to show statistical significance. It appears that treatment with oxytetracycline, streptomycin, or pencillin had neither a beneficial nor a harmful effect on semen production or quality when given to rabbits considered to be normal.

TABLE 2—Survival Time of Rabbit Sperm During Storage (Days)

Collection	Group 1 (oxytetracycline)	Group 2 (penicillin)	Group 3 (streptomycin)	Group 4 (control)
Before treatment average	10.1	12.9	12.6	12.6
During treatment				
1st week	12.0	12.0	12.0	11.3
2nd week	13.3	13.3	16.0	17.3
3rd week	11.3	12.0	10.7	12.0
Average	12.2	12.4	12.9	13.6
After treatment				
1st week	17.3	19.3	18.0	13.3
2nd week	12.0	17.3	17.3	18.0
3rd week	16.7	14.7	16.7	16.0
4th week	14.0	13.3	14.0	14.7
5th week	10.7	13.3	11.3	12.0
Average	14.1	15.6	15.5	14.8

General observations of the rabbits revealed that those which received oxytetracycline had a rough hair coat at the end of the treatment period. Also, those receiving penicillin were inappetent at the end of the treatment period. Some diarrhea occurred in the rabbits in treated groups, but this was not serious.

Body temperatures were checked once daily in the morning for one week before the treatment and for five weeks after treatment. During the treatment period, it was checked twice daily, once before injection and again about one and one half hours after the injection. The body temperature did not change noticeably following administration of the antibiotics.

DISCUSSION

Therapeutic doses of oxytetracycline, streptomycin, and penicillin did not appear to have any beneficial or harmful effect on semen production as (1) indicated by volume of semen, sperm concentration, and sperm per ejaculation, or (2) as measured by motility, per cent alive, per cent abnormal, and survival of sperm during storage. These observations are in agreement with the findings of Carpenter and Larson,[1] Catron,[2] and De Pape *et al.*,[4] who observed no effects on swine reproduction from feeding antibiotics. They are also in agreement with the studies by Rusoff *et al.*,[8] in which they found no alteration in quality of semen from bulls fed 300 mg. of chlortetracycline daily. The study by Uram *et al.*,[11] in which antibiotics were fed to rats without altering reproduction, also agrees with the present study and with the general observations that have been made.

The present study differs from the previous research in that antibiotics were injected intramuscularly at therapeutic levels rather than fed at levels recommended for increasing the rate of weight gain. Greater

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amounts were administered and destruction and loss from the gastrointestinal tract did not ensue. Thus, the present study employed therapeutic antibiotic levels over a relatively long period, which differs both from the feeding experiments previously cited and from the work of Sciortino,[9] who used a single injection of 3,600,000 to 7,690,000 units of penicillin for rabbits. His results on histology of the testes are in agreement with the present study.

Since the injection of penicillin, oxytetracycline, and streptomycin at therapeutic levels into normal rabbits over a period of three weeks had no apparent beneficial or harmful effects on semen production or quality, it would appear that these antibiotics are without direct effects on reproduction. Animals in which fertility is impaired following the recovery from illness in which antibiotics have been used may be explained on the basis that the reduced fertility resulted from the illness rather than the antibiotics. Also, the improvement in fertility following the use of antibiotics to assist in correcting a diseased condition probably is attributable to correcting the diseased condition which was causing impaired fertility, rather than the induction of greater fertility by the antibiotics.

Summary and Conclusions

Twelve New Zealand White male rabbits, about 5 months old, were used to study the effect of antibiotics on semen production. They were allotted randomly to 4 groups. Those in group 4 served as controls, and those in groups 1, 2, and 3 were daily injected intramuscularly with therapeutic doses of Terramycin (oxytetracycline), penicillin, and streptomycin (2 mg. of oxytetracycline, 4,000 units of procaine penicillin, and 40 mg. of streptomycin in aqueous solution per kilogram of body weight daily, respectively for three weeks).

Semen collections were made every seven days during the pretreatment period of two months, during the three weeks of treatment, and for five weeks after the treatment was discontinued. The semen was examined for volume, motility, concentration, percentage of live sperm, percentage of normal sperm, and survival time during storage. None of the antibiotics caused a definite change in the semen volume, percentage of live sperm, or percentage of abnormal sperm, nor did they alter significantly the survival time of spermatozoa in stored rabbit semen.

It appears that antibiotics cause no beneficial or harmful effects on semen production or quality. Decreased fertility following the use of antibiotics given to correct a disease condition is probably attributable to damage caused by the disease. Increased fertility following the use of antibiotics therapeutically is probably attributable to eliminating the disease which was causing impaired fertility.

References

[1] Carpenter, L. E., and Larson, N.: Antibiotics and the Reproduction of Swine. J. Anim. Sci., *12*, (1953): 812.

[2] Catron, Damon: A 1953 Appraisal of Antibiotics for Swine. Proc. 4th Ann. Nutritional School for Feed Men, (1953): 33–41.

[3] Davey, Robert J., and Stevenson, J. W.: Comparison of Effects of Levels of Aureomycin on Reproduction in Swine. J. Anim. Sci., *12*, (1953): 912.

[4] De Pape, J. G., Burkitt, W. H., and Flower, A. E.: Dehydrated Alfalfa and Antibiotic Supplements in Gestation-Lactation Rations for Swine. J. Anim. Sci., *12*, (1953): 77.

[5] Lambert, W. V., and McKenzie, F. F.: Artificial Insemination in Livestock Breeding. U.S.D.A. Circular 547, U.S. Government Printing Office, Washington, D.C. 1940.

[6] Mayer, D. T., Squiers, C. D., Bogart, R., and Oloufa, M. M.: The Technique for Characterizing Mammalian Spermatozoa as Dead or Living by Differential Staining. J. Anim. Sci., *10*, (1951): 226–235.

[7] Perry, Enos T.: The Artificial Insemination of Farm Animals. 2nd ed. Rutgers University Press, New Brunswick, N.J., 1955.

[8] Rusoff, L. L., Hag, M. O., Branton, Cecil, Patrick, T. E., and D'Arensbourg, G. D.: Report on the Feeding of Aureomycin Supplement to Mature Dairy Bulls and Lactating Dairy Cows. J. Anim. Sci., *11*, (1952): 774.

[9] Sciortino, G.: The Action of Antibiotics on Spermatogenesis. Boll. Soc. ital. Biol. sper., *26*, (1950): 1605–1606.

[10] Thacker, D. L., and Almquist, J. O.: Diluter for Bovine Semen: Fertility and Motility of Bovine Spermatozoa in Boiled Milk. J. Dai. Sci., *36*, (1953): 173–180.

[11] Uram, J. A., French, C. E., Barron, G. P., and Swift, S. W.: The Effect of High Levels of Terramycin and Streptomycin on Growth, Reproduction and Lactation of the Rat. J. Nutrition, *55*, (1955): 481.

[12] Wu, S. H., Elliker, P. R., and McKenzie, F. F.: The Microorganisms in Dairy Bull Semen and Their Relation to Fertility. Oregon Agric. Exper. Station Tech. Bull. 24, 1952.

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A Study of Infertility in Cattle in Alabama

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INFERTILITY in cattle has long been a serious problem. Specific diseases such as brucellosis, vibriosis, trichomoniasis, and leptospirosis still cause great economic loss, but they can be detected with reasonable accuracy and controlled or eliminated.

Nonspecific or "unspecialized" infections, functional disorders, endocrine dysfunctions, nutrition, and heredity have all added to the complexity of the picture. One of the most perplexing and discouraging problems in a herd in which most of the animals breed normally is the infertile cow with a normal estrous cycle and no apparent pathological changes. In our experience, approximately 5 to 8 per cent of the animals of a given herd may be placed in this category.

REVIEW OF LITERATURE

Many authors [3, 11, 17, 26, 27] point out the multitude of factors that may be involved in bovine breeding failures. These include poor management, inadequate nutrition, hormone imbalance, anatomic abnormalities, genetic aberrations, pathological changes, and infections. The latter includes specific and nonspecific infections which involve a variety of infectious agents. It has been shown that the uteri of normal animals are usually bacteriologically sterile.

Reports [7, 14–16, 23] indicate that bacterial infection, either clinically apparent or not apparent, may be responsible for many cases of infertility. The bacteria most often involved are micrococci, streptococci, corynebacteria, Neisseria organisms, and diphtheroids. Alford *et al.*[1] isolated a group A Streptococcus as a cause of infertility in one herd. Edward *et al.*[6] reported the isolation of pleuropneumonia-like organisms from the bovine genital tract. Millar [25] reported the transmission of a filterable virus capable of inducing an endometritis in cattle. Other workers [12, 22, 28, 30] have found molds associated with the bovine genital tract, some of which were definitely shown to produce abortion. Members of the family Mucoraceae were most often responsible for the infections.

Durrell [9] made a study of the relationship of nutrition to infertility in dairy cattle in Canada and concluded that malnutrition, *i.e.*, protein deficiency, low energy intake, low plane of nutrition, or inadequate levels of vitamins A and C, was the most prevalent predisposing cause of sterility. Hignett and Hignett [19] showed that there is a definite relationship between P_2O_5 intake and fertility, and that there is a similar relationship between CaO and fertility when the P_2O_5 intake is either low or high.

Filmore [13] discussed a variety of inheritable factors that cause reproductive inefficiency in cattle. Olds [26] indicated that inheritance of low fertility and close-breeding practices may account for much of the infertility in dairy herds. Gibbons,[11] however, does not think that heredity is an important factor in the over-all picture.

Although endocrine disturbances are considered by many to be a cause of reduced fertility, Asdell,[3] Frank,[8] and Roberts [27] found that various hormonal treatments were not effective. The percentage of response was essentially the same, regardless of what hormone was used.

The consensus seems to be that the majority of breeding troubles in cattle are the result of infection that may or may not produce visible pathological changes but that either destroys the fertilized egg or causes early embryonic death.[3–5, 11, 15, 20, 26, 27, 33] Mild irritants, antibiotics, sulfonamides, antiseptics, buffer-nutrient solutions, "soda" douches, and hormones have all been used therapeutically with varying success.[4, 5, 10, 18, 20, 21, 24, 34]

Most writers claim to have good to excellent results with the treatments they are using—the range of effectiveness being from 40 to 90 per cent. However, Ulberg *et al.*[33] got lower conception rate in animals treated with antibiotics than in their control animals. They also found that if the uterus of a cow was infused with antibiotics 17 days following estrus, then recovered at necropsy 16 to 48 hours later, it would contain yellow, cheesy exudate, epithelial desquamation, and leukocytic infiltration. If the uterus was infused at the time of estrus, no pathological changes were found.

The work of Simon and McNutt [29] also tends to refute the generally accepted assumption that most infertility is caused by infection of the uterus. They found that the endometria of affected cows did not differ greatly from that of apparently normal heifers and that endometritis was not a major cause of infertility in otherwise apparently normal cows.

This is a report of (1) result of cultures taken from cattle in problem herds; (2) pathological conditions found in the uteri

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of a limited number of sterile cattle at the time of slaughter; (3) normal cattle that have had their genital tracts infused with bacteria isolated from infertile cows; and (4) treatments that have been used on infertile ("repeat-breeder") cattle. Particular attention was paid to breeding problems not associated with specific diseases.

Materials and Methods

Experiment 1.—Between Oct. 10, 1956, and June 30, 1958, culture specimens were obtained on 562 trips to 32 farms in various parts of Alabama, five substations of the Agricultural Experiment Station, and from three cattle units at the Alabama Polytechnic Institute. Specimens were collected from cows referred to the large animal clinic, School of Veterinary Medicine, because of infertility. Before work was started on the animals, an over-all herd survey was made whenever possible. Information was obtained relative to the number of animals in the herd, the type of housing, feeding practices, tests for tuberculosis and brucellosis, the present breeding status of the herd, and the occurrence of parturient diseases during the previous year. The breeding histories of individual cows in question were also obtained when available.

Cultures were taken from the cervices of cows bred three or more times without conceiving. The perineal region was thoroughly washed, flushed with an antiseptic solution, and dried with paper towels. The lips of the vulva were then parted and a glass speculum 1 inch in diameter and 18 inches long, containing a long sterile cotton swab, was inserted into the vagina to the cervix. Cervical secretion was secured with the swab and streaked on blood agar and Sabouraud's mediums. The swab was then broken from the wooden applicator stick aseptically and allowed to fall into a vial of thiol medium. Inoculated mediums were placed in a portable incubator and taken back to the laboratory, where the thiol medium was incubated under CO_2 tension in a candle jar.

At the time of culture, a thorough examination was made of the cervix and vagina with the aid of a long-necked inspection light and a speculum. The genital organs were also palpated rectally to determine if any palpable lesions or anatomic deformity was present. Whenever indicated, blood samples were collected for the brucellosis agglutination test and the microscopic agglutination test for leptospirosis. If the cows had aborted, the genital organs were examined and cultures were taken; if the fetus was in suitable condition, the body fluids and stomach contents were cultured and sections of the liver, spleen, and kidneys were taken for microscopic examination. Whenever possible, the genital organs of infertile cattle were obtained at the time of slaughter, cultured, and examined both macroscopically and microscopically.

All recovered bacteria considered pathogenic by the usual laboratory standards were lyophilized and stored for future use. During the first part of the experiment, all hemolytic streptococci were classified according to their action in the various mediums. Later, they were typed serologically.

Experiment 2.—In 1956, 12 sexually mature virgin heifers were obtained as experimental animals. The cervices of these animals were cultured three times at weekly intervals to be sure that they harbored no bacterial infection. Nine of the heifers were inoculated at the external os of the cervix: 3 with a 2-day-old culture of *Streptococcus pyogenes* isolated from an infertile cow; 3 with a culture of Pneumococcus isolated from an animal with a case of chronic metritis; and 3 with a coagulase-positive *Staphylococcus aureus* culture. Three cows were left as uninoculated controls. Each heifer was bred artificially at a subsequent heat period after a culture was made from the cervix. All heifers were similarly inoculated with the original bacteria four and one half months after the first inoculation. At that time, all of the heifers except 1 were pregnant. Sterile saline solution was used as an inoculum for the controls.

One heifer from each group was inadvertently infected with *Vibrio fetus* by the use of semen from an infected bull. The infections were detected at the next service and *V. fetus* was isolated from the bull's semen.

After each heifer had freshened and passed the fetal membranes (all within 24 hr.), 10 cc. of a broth culture of the same bacteria with which the heifer was previously inoculated was introduced directly into the uterus. For this purpose, 48-hour cultures in brain-heart infusion broth were used. It was thought that if the bacteria in question were responsible for infertility, they would become established in an organ that was vulnerable because of recent trauma.

Experiment 3.—The cervices of approximately 16 cattle were cultured for pleuropneumonia-like organisms (PPLO) in addition to the regular routine culturing. Of the 16 animals, 7 (44%) were positive for this organism, which was grown in Difco PPLO broth without crystal violet. The presence of organisms was determined by plating on PPLO agar and examining the surface of the agar under a wide-field microscope. Subsequent to culturing, the organisms were passed four times in chicken embryos in an attempt to produce changes in the embryo. Prior to use in calves, one of the isolates was again grown in broth. A 4-cc. dose of this broth culture was inoculated intravenously into each of 2 calves—1 calf was 6 days old and the other 12 days old. The calves were observed closely for two weeks and their temperatures were taken daily.

Experiment 4.—Many of the cattle examined were treated with antibiotics in an effort to determine whether this treatment had any beneficial effects on cattle that presented breeding problems and, if so, which preparation would be most efficacious. Five different preparations were infused into the uteri: (1) 200,000 units of crystalline penicillin and 1 Gm. of streptomycin; (2) 16,000

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units of polymyxin sulfate and 800 units of bacitracin (Polycin-Soluble Tablets*) dissolved in 10 cc. of sterile distilled water; (3) 1 Gm. of intramuscular-type chloramphenicol; (4) 100 mg. of tetracycline hydrochloride, intravenous type (Panmycin†); and (5) 400 mg. of tetracycline hydrochloride in the form of soluble powder. Most of the infusions were made four to 24 hours after service; however, a few (21) were made during anestrus. Seven others were made about six hours prior to breeding.

RESULTS

Experiment 1.—A total of 1,359 isolation attempts were made. The type and number of bacteria isolated from the genital tracts (table 1) and from aborted fetuses of cows (table 2) are shown. Twenty-four sets of genital organs were obtained from infertile cows at necropsy. Bacteria could be isolated from only 7, and 13 showed no pathological changes (table 3).

TABLE 1—Bacteria Isolated from the Cervices and Anterior Portions of the Vaginas of Cattle

Organisms	No. isolated
Bacillus subtilis	29
Escherichia coli	45
Vibrio fetus	25
Other Vibrio sp.	30
Gaffkya tetragena	10
Gram-positive rods	14
Gram-negative rods	5
Sarcina lutea	1
Diplococcus pneumoniae	2
Staphylococcus	
Nonhemolytic *Staph. aureus*	17
Hemolytic *Staph. aureus*	
Coagulase neg.	10
Coagulase pos.	2
Nonhemolytic *Staph. albus*	60
Hemolytic *Staph. albus*	5
Streptococci	
Animal C	6
Human C	4
Group E	38
Group G	10
Group H	1
Pyogenes	12
Agalactiae	2
Viridans	1
Unclassified (*beta*-hemolytic)	20
Anaerobic	7
Nonhemolytic	11
Clostridium sp.	1
Proteus sp.	6
Corynebacterium sp.	2
Pleuropneumonia-like organisms	7
Negative	976
Total No. isolation attempts	1,359

Experiment 2.—Bacteria which are usually classified as pathogens (*Str. pyogenes*, Pneumococcus, and *Staph. aureus*) had no apparent effect on breeding efficiency when used to inoculate the external ora of the cervices of virgin heifers before breeding. Six of the 12 heifers required an average of two services, whereas 2 control heifers required one and two services, respectively. Inoculation of the same bacteria at the external os of the cervix after the animal conceived had no effect on the state of pregnancy.

TABLE 2—Bacteria Isolated from 28 Aborted Fetuses

Organisms	No. isolated
Streptococci	1
Coliform	4
Vibrio fetus	5
Leptospira pomona	1
Negative	17
Total	28

Only 4 heifers that had been accidentally infected with *V. fetus* presented breeding difficulties. These animals required an average of 5.75 services (table 4). Statistically, there was a high level of significance between the number of services required for conception in those heifers infected with *V. fetus* (4) as compared with those not infected with *V. fetus* (8), regardless of the presence or absence of the other types of bacteria.*

When these heifers were inoculated in the uterus after calving (1957–58), they required an average of 3.2 services for conception, whereas the 3 control animals required 3.0 services under the same environmental conditions (table 5). Biometric analysis indicated that in this phase of

TABLE 3—Necropsy Findings in Genital Organs of Infertile Cattle

Condition	No. organs
BACTERIOLOGICAL FINDINGS	
Vibrio sp.	1
Staph. albus (coag. neg.)	4
Staph. aureus	1
Streptococcus (nonhemolytic)	1
Negative	17 (71%)
PATHOLOGICAL FINDINGS	
Cystic corpus luteum	6
Slight fibrosis, endometrium	3
Cystic follicle	2
Mild endometritis	2
Slight epithelial hyperplasia, fallopian tubes	2
Ovarian adhesions, hydrosalpinx	1
No pathological changes	13 (54%)
Total No. examined	24

* Polycin-Soluble Tablets are produced by Pitman-Moore Co., Division of Allied Laboratories, Indianapolis, Ind. † Panmycin is produced by the Upjohn Co., Kalamazoo, Mich.

* Statistical analysis was made by Dr. Albert E. Drake, associate professor and associate biometrician, Alabama Polytechnic Institute, Auburn.

TABLE 4—Breeding Efficiency of Heifers After Pathogenic Bacteria Were Inoculated at External Os of Cervix Prior to Breeding, 1956–1957, and into the Uterus After Calving, 1957–1958

Heifer No.	Bacteria	Services required for conception after inoculation	
		At external os	In uterus
70	*Str. pyogenes*	4	3
73	*Str. pyogenes* (Vibrio)	3	1
75	*Str. pyogenes*	2	4
76	Control	2	5
78	Control (Vibrio)	6	3
81	Control	1	1
80	Pneumococcus (Vibrio)	6	3
82	Pneumococcus	2	2
83	Pneumococcus	1	1
84	*Staph. aureus*	2	5
85	*Staph. aureus*	1	8
87	*Staph. aureus* (Vibrio)	8	2

the experiment the presence or absence of "pathogenic" bacteria in the genital tract had no significant effect on the breeding efficiency of the animals. Those inoculated in the uterus after calving required more services than for their first conception. The controls, however, also required more services than during their first breeding period, the number closely approaching that of the inoculated animals.

When cultures were taken at each heat period, the bacteria placed either at the external os of the cervix or in the uterus could not be recovered at the cervix at any time.

Experiment 3.—The strain of PPLO isolated from an infertile cow and inoculated intravenously into 2 young calves produced no disease. The animals remained normal throughout the experiment.

Experiment 4.—Results obtained with intrauterine infusions of antibiotics are shown (table 5). Many more animals were treated than is indicated. Only those treatments in which the results were certain are included in the table.

DISCUSSION

Although the main objective of the first phase of this work was to take cultures from infertile cattle, in a few instances cultures were obtained from animals in herds where it was later shown that management may have played an important part in bringing about breeding difficulties. Also, routine cultures were attempted from animals in herds in which sterility work was being done.

Of 1,359 cultures taken from the external ora of the cervices of cattle, 383 showed the presence of bacteria. It is felt that most of the bacteria cultured were opportunists and only transient in nature. It was rare that the same bacteria could be cultured from an animal on two successive occasions. An example of the variety of organisms isolated on successive attempts from a single cow is as follows: (1) Staphylococcus, (2) *Aerobacter aerogenes*, (3) negative, (4) Streptococcus, group E, (5) negative, (6) Streptococcus, group E, (7) negative, (8) nonhemolytic *Staphylococcus albus*, (9) negative, and (10) nonhemolytic *Staph. albus* and Proteus.

Except for the few heifers with a small amount of pus present or a slight cervicitis, no gross pathological changes were observed in the animals examined.

The placing of pathogenic bacteria into the genital tracts of clean heifers apparently had no effect on their breeding efficiency. The accidental introduction of *V. fetus* did, however, markedly increase the number of services required for conception.

These animals were bred the second time during the fall and winter of 1957–1958, at a time when the weather was the coldest in many years. Feed consisted of poor-quality hay, ground snapped corn, cottonseed meal, and mineralized salt. During the previous summer, suitable pasture land was not available and the animals had lost considerable weight. It is believed that the low level of nutrition and severe weather conditions played an important part in

TABLE 5—Breeding Efficiency of Cows Given Intrauterine Infusions of Antibiotics

Antibiotic	No. animals treated	No. conceived			Did not conceive	
		At treatment	Next heat	Subsequent heat	No.	%
Penicillin-streptomycin	21	7	6	1	7	33.3
Polymyxin-bacitracin	40	17	6	10	7	17.5
Chloramphenicol	34	8	8	5	13	38.2
Tetracycline HCl (100 mg.)	8	0	1	3	4	50.0
Tetracycline HCl (400 mg.)	36	6	11	11	8	22.2
Total	139	38	32	30	39	28.1

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increasing the number of services per conception.

No lesions were found in 13 of the 24 sets of bovine genital organs examined. In one, there were ovarian adhesions and hydrosalpinx. Microscopically, the uteri of 3 cows from the college herd appeared to show mucosal fibrosis which may have been the result of students practicing artificial insemination. Two of the cows had a low-grade endometritis and 2 had a slight epithelial hyperplasia of the fallopian tubes. In 1 cow, the endometritis was associated with a follicular cyst and a cystic corpus luteum. Most of the trouble seemed to be in the ovaries, with six cystic corpora lutea and two cystic follicles.

Bacteria were isolated from 7 of the 24 uteri. With the possible exception of the Vibrio, these bacteria were considered nonpathogenic. This seems to be in agreement with the work of Simon and McNutt,[29] who indicated that endometritis was not a major cause of infertility. It would appear that a hormonal or physiological dysfunction may have been involved in a large percentage of the cases.

Roberts[27] stated that the chances of a cow conceiving are 50 per cent at the third service, 40 to 45 per cent at the fourth service, and 30 to 35 per cent at the fifth and sixth services. Therefore, it appears (table 5) that the treatments used were not effective in controlling infertility. In this series, cattle were treated in a number of herds throughout Alabama under differing environmental conditions, as well as at the large animal clinic. Since control animals were not used, it should be assumed that any effective treatment of infertile cows must raise the number of those that conceive on the next service to over 50 per cent.

SUMMARY AND CONCLUSIONS

1) In 1,359 attempts, bacteria were isolated from the genital organs of cows on 383 (28.1%) occasions.

2) Breeding efficiency was not affected when a *Streptococcus pyogenes* and a coagulase-positive *Staphylococcus aureus* isolated from "repeat breeder" cows, and a Diplococcus organism from a cow with chronic metritis, were used to inoculate the cervices or the uteri of experimental heifers.

3) A pleuropneumonia-like organism isolated from an infertile cow was not pathogenic to 2 young calves when inoculated intravenously.

4) Routine intrauterine infusions of antibiotics did not affect the breeding efficiency of cattle.

5) "Nonspecific" bacterial infections did not appear to be the major cause of infertility in the cattle studied.

6) Many of the bacteria isolated were considered to be opportunists.

7) It is postulated that some hormonal or physiological dysfunction or possibly a nutritional factor was a major cause of infertility in the animals studied.

References

[1] Alford, J. A., Gunter, J. J., and Edwards, C. D.: Reproductive Tract Infection in a Dairy Herd Caused by Group A Streptococci. Cornell Vet., *45*, (1955): 357.

[2] Asdell, S. A.: Hormones and the Treatment of Sterility in Dairy Cattle. J. Dai. Sci., *32*, (1949): 45.

[3] Bartlett, D. E.: Infectious Diseases as a Cause of Infertility. J. Dai. Sci., *32*, (1949): 36–44.

[4] Chambers, E. E.: Penicillin in the Treatment of Non-breeding Cows. North Am. Vet., *29*, (1948): 640–641.

[5] Chambers, E. E.: Treatment of Conditions Affecting Bovine Fertility. Vet. Med., *47*, (1952): 128–132.

[6] Durrell, W. B.: A Survey of the Role of Nutrition in Sterility of Dairy Cattle. Canad. J. Comp. Med. & Vet. Sci., *15*, (1951): 1–11.

[7] Easley, G. T., Leonard, R. H., and Trotter, D. M.: Bacteriological, Pathological and Clinical Studies of the Reproductive Tract of the Hereford Cow and a Bacteriological Study of Hereford Bull Semen. North Am. Vet., *32*, (1951): 258–266.

[8] Edward, D. G., Hancock, J. L., and Hignett, S. L.: Isolation of Pleuropneumonia-like Organisms from the Bovine Genital Tract. Vet. Rec., *59*, (1947): 329–330.

[9] Frank, A. H.: Impaired Breeding in Cattle. Field Observations and Results of Treatment. Proc. Book, AVMA (1950): 190.

[10] Fincher, M. G.: Antibiotics in Sterility. Vet. Med., *37*, (1952): 185–188.

[11] Gibbons, W. J.: The Problem of Infertility of Cattle. North Am. Vet., *38*, (1957): 257.

[12] Gilman, H. L., and Birch, R. R.: A Mold Associated with Abortion in Cattle. Cornell Vet., *16*, (1925): 81–89.

[13] Gilmore, L. O.: The Inheritance of Functional Causes of Reproductive Inefficiency—A Review. J. Dai. Sci., *32*, (1949): 71–91.

[14] Gunter, J. J., Collins, W. J., Owen, J., Sorensen, A. M., Scales, J. W., and Alford, J. A.: A Survey of the Bacteria in the Reproductive Tract of Dairy Animals and Their Relationship to Infertility. Am. J. Vet. Res., *16*, (April, 1955): 282–285.

[15] Hardenbrook, H., Jr.: The Diagnosis and Treatment of Non-specific Infections of the Bovine Uterus and Cervix. J.A.V.M.A., *132*, (June 1, 1958): 459.

[16] Hatch, R. D., Feenstra, E. S., and Jennings, L. F.: A Bacteriological Survey of the Reproductive Tract of Infertile Cows. J.A.V.M.A., *114*, (March, 1949): 131–133.

[17] Herrick, J. B.: Field Observations on Bovine Infertility. North Am. Vet., *36*, (1955): 365.

[18] Herrick, J. B.: Clinical Observations on the Use of Intrauterine Infusions. North Am. Vet., *36*, (1955): 545.

[19] Hignett, S. L., and Hignett, P. G.: The Influence of Nutrition on Reproduction Efficiency in Cattle. I. The Effect of Calcium and Phosphorus Intake on Fertility of Cows and Heifers. Vet. Rec., *63*, (1951): 603–609.

[20] Hinze, P. M.: Infertility in Dairy Herds. Vet. Med., *47*, (1952): 397.

[21] Huber, W. G.: A Breeding-Rest Fertility Program. Vet. Med., *52*, (1957): 585.

[23] Jungherr, E.: Mycotic Affections of the Bovine Reproductive System. J.A.V.M.A., *86*, (Jan., 1935): 64–75.

[24] Lindley, D. C., and Hatfield, R. C.: Observations on the Bacterial Flora of Infertile Dairy Cows. J.A.V.M.A., *120*, (Jan., 1952): 12–15.

[25] Lindley, D. C.: Intrauterine Antibiotic Therapy Postservice in Infertile Dairy Cattle. J.A.V.M.A., *124*, (March, 1954): 187–189.

[26] Millar, P. G.: Viral Infertility in Cattle. Brit. Vet. J., *111*, (1955): 309.

[27] Olds, D.: Infertility in Cattle—A Review. J.A.V.M.A., *122*, (April, 1953): 276–286.

[28] Roberts, S. J.: An Evaluation of Uterine Infusions for the Treatment of Infertility in Cattle. Cornell Vet., *46*, (1956): 21–38.

[29] Rollinson, D. H. L., and Haq, I.: Mycotic Infection of the Prepuce of the Bull. Vet. Rec., *60*, (1948): 69–70.

[30] Simon, J., and McNutt, S. H.: Histopathological Alterations of the Bovine Uterus. II. Uterine Tissue from Cows of Low Fertility. Am. J. Vet. Res., *18*, (April, 1957): 241–245.

[31] Smith, T. J.: Mycosis of the Bovine Foetal Membranes. J. Exptl. Med., *31*, (1920): 115–122.

[32] Tanabe, T. Y., and Casida, L. E.: The Nature of Reproductive Failures in Cows of Low Fertility. J. Dai. Sci., *32*, (1949): 237.

[33] Tanabe, T. Y., and Almquist, J. O.: Some Causes of Infertility in Dairy Heifers. J. Dai. Sci., *36*, (1953): 586.

[34] Ulberg, L. C., Black, W. G., Kidder, H. E., McDonald, L. E., Casida, L. E., and McNutt, S. H.: The Use of Antibiotics in the Treatment of Low Fertility Cows. J.A.V.M.A., *121*, (Dec., 1952): 436–440.

[35] Woelffer, E. A.: A Treatment for Shy Breeding Cows. J.A.V.M.A., *116*, (June, 1950): 425–426.

[36] Woelffer, E. A.: Some Problems of Infertility in Cattle. Vet. Med., *46*, (1951): 253–256.

XUM

Scientific Notes

Incidence of Certain Gastrointestinal Parasites of Cattle in Texas

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In a preliminary study to find a more satisfactory method of control of gastrointestinal parasites of cattle in Texas, it was found necessary to ascertain the most common and most pathogenic parasites present.

Woodhouse and Reid,[1-2] working at Angleton, Texas, identified several genera of parasites from cattle. However, the cattle came from the Gulf Coast region only. Therefore, a survey of cattle parasites was made from several regions in the state in an effort to determine the effect of rainfall, temperature, and other varying climatic conditions on the parasite population and distribution.

Materials and Methods

The abomasums and small intestines were collected from cattle at eight locations in north central and northeastern Texas (fig. 1). To increase the probability that all were from native cattle, materials were collected at small local slaughter plants.

No selection of animals was made except to collect organs from adults, yearlings, and calves at each location where possible. Frequently, it was impossible to obtain viscera from all three age groups at each location. The approximate age was determined at the time of slaughter. The viscera of milk-fed calves were not examined. Most of the animals were in fair to good condition. The rumen and large intestine were not routinely examined. Collection of material extended from January, 1957, to November, 1957.

Fig. 1—Counties in which collections of gastrointestinal parasites were made.

TABLE 1—Average Number of Parasites Found by Age of Animal

Age	Animal (No.)	Haemonchus sp.	Ostertagia sp.	Trichostrongylus sp.	Cooperia sp.*
ABOMASUM					
Adult	6	725	1,790	430	15.0
Yearling	53	165	654	1,004	1.6
Calves	41	205	631	368	0
Total	100	214	712	719	0

Age	Animal (No.)	Cooperia sp.	Trichostrongylus sp.	Nematodirus sp.	Ostertagia sp.*	Bunostomum sp.*
SMALL INTESTINE						
Adult	6	719	0	0	0	0
Yearling	53	2,656	67	60	0.54	0
Calves	41	859	16	8	0	2.6
Total	100	1,803	42	35	0	0

* Worms of this genus were found in only 1 animal of the age group indicated.

Procedure

At the time of slaughter, the abomasum was dissected from the omasum and tied at the omasal and pyloric ends. The small intestine was tied at the pylorus and ileocecal junction. These viscera were excised, placed in cans, iced, and returned to the laboratory. At the laboratory, the tracts were dissected, separated, placed in separate containers, and thoroughly washed in a known volume of water. An aliquot was taken of the washings, placed in jars, and sufficient formalin was added to form a concentration of 10 per cent.

For examination, these specimens were placed in Petri dishes with concentric circles drawn on

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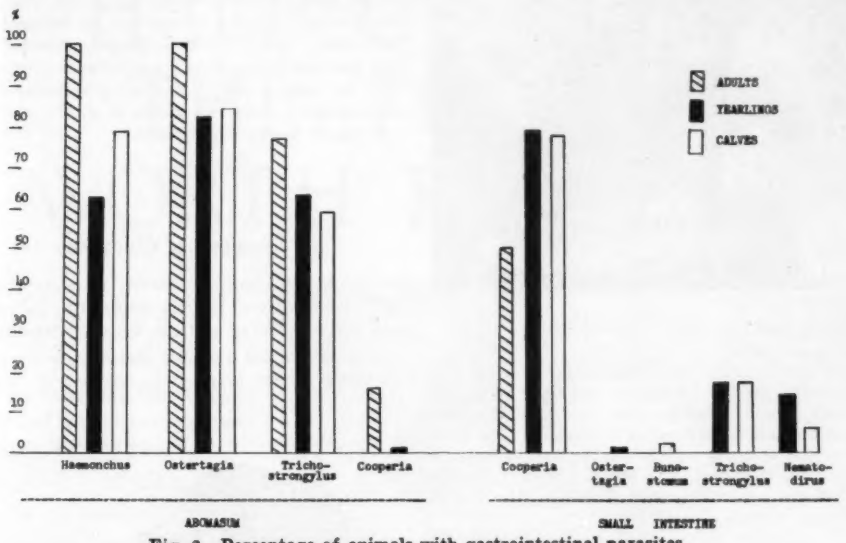

Fig. 2—Percentage of animals with gastrointestinal parasites.

the bottom. A dissecting microscope was used to locate the worms. All the parasites in the aliquot were counted. Generic identifications were made on the first 100 worms observed (or on all contained in the sample). No attempt was made to identify species. The tapeworms were counted and identified individually.

RESULTS

The average number of parasites per animal by genera is reported (table 1). The figures for Cooperia sp. in the abomasum, and Ostertagia sp. and Bunostomum sp. in small intestines, represent parasites found in only 1 animal in each age group.

The percentages of animals infected in each age group, and the genera of parasites are shown graphically (fig. 2).

References

[1] Woodhouse, C. A., Reid, J. J., and Schmidt, Hubert: Investigation of Gastro-Intestinal Parasites in Cattle. 53rd Ann. Rep. Texas Agric. Exper. Sta. (1940): 159–160.

[2] Woodhouse, C. A., Reid, J. J., and Schmidt, Hubert: Investigation of Gastro-Intestinal Parasites in Cattle. 54th Ann. Rep Texas Agric Exper. Sta. (1941): 111-112.

Symposium on Antibiotics

The Seventh Annual Symposium on Antibiotics, sponsored by the journals *Antibiotics & Chemotherapy* and *Antibiotic Medicine & Clinical Therapy*, will be held Nov. 4–6, 1959, at the Mayflower Hotel, Washington, D.C.

Papers presented at this meeting will be published in "Antibiotics Annual 1959–1960." Authors interested in contributing may obtain information on manuscript preparation by contacting Dr. Henry Welch, Director, Division of Antibiotics, Food and Drug Administration, U.S. Department of Health, Education, and Welfare, Washington 25, D.C.

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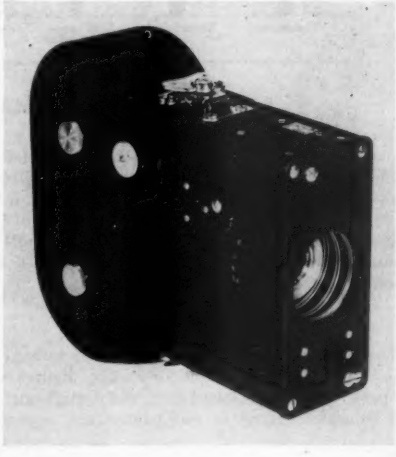

can be controlled manually with a push-button switch. A selector switch on the magazine controls single or double frame advance; frame speed is up to six frames per second. The camera has a film capacity of 100 ft. and a maximum shutter speed of 1/100 sec. It has rechargeable battery power or can be operated on regular A.C. power.

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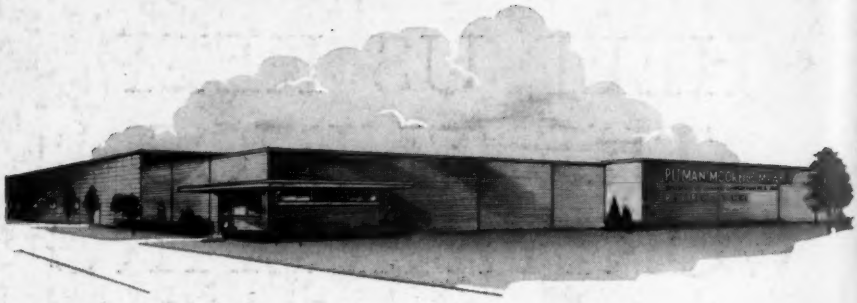

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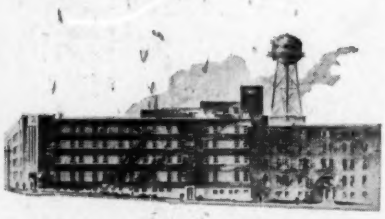

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